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REMARKS

Status of the Claims

Claims 7-9 and 11-12 have been amended without prejudice to or disclaimer of the subject matter therein to recite and as described elsewhere herein. Support for these amendments is found in the original claims. Claims 7 and 11 have been further amended to recite "in the range of 3.0-3.5 M salt," support for which can be found on page 9, line 14. Claim 11 has been further amended to correct the inclusion of "between," support for which can be found in the original claim. Claims 12 and 14-16 have been amended to recite "substantially homogeneous type II-like collagen from one or more species of jellyfish...." Support for these amendments are found in original claim 1 and in the specification at page 7, lines 13-28. Therefore, no new matter has been added by amendment. Claims 1-16 are now pending.

The Examiner's comments are addressed below in the order set forth in the Office Action.

The Objections to the Specification Should Be Withdrawn

The Office Action objects to the inclusion of attorney docket numbers and document identifiers within the specification. Applicant requests clarification of the basis for this objection. Applicant earnestly believes that such footers are permissible and respectfully requests that the objection be withdrawn.

The Rejections of the Claims Under 35 U.S.C. § 112, 2 Should Be Withdrawn

Claims 1-14 and 16 stand rejected under 35 U.S.C. § 112, 2. Applicant respectfully traverses.

The Office Action asserts that the term "substantially homogeneous" is indefinite and that Applicant must specify a degree of purity or specifically recite the specification definition. As noted in the Office Action, the term has been defined on page 7, lines 13-28 of the specification to mean the following.

> By "substantially homogeneous" type II-like collagen is intended type II-like collagen molecules in the substantial absence of other biological macromolecules, e.g., other contaminating types of collagen, polynucleotides, proteins, etc. Preferably a substantially homogeneous jellyfish collagen is at least

> 85% by weight (wt %), more preferably at least 90% by weight, most preferably at least 95%, 96%, 97%, 98%, 99% or greater by weight of the indicated biological macromolecules present. However, the term "biological macromolecules" does not include water, buffers, and other small molecules, especially molecules having a molecular weight of less than 1000 daltons. In no case does a substantially homogeneous type II-like jellyfish collagen encompass the collagen present in its natural source.
>
> A substantially homogeneous jellyfish collagen is substantially free of natural contaminants. By collagen that is "substantially free of natural contaminants" is intended collagen that has, at some point, been purified to be substantially homogeneous. However, collagen that is substantially free of natural contaminants may be associated with other compounds. In no case does collagen substantially free of natural contaminants encompass the collagen present in its natural source.

The legal standard of definiteness is whether a claim reasonably apprises those of skill in the art of its scope. *See In re Warmerdam*, 33 F.3d 1354, 31 USPQ2d 1754 (Fed. Cir. 1994). The claim must be read in light of the specification. *See, e.g., Credle v. Bond*, 25 F.3d 1566, 30 USPQ2d 1911 (Fed. Cir. 1994). Given the guidance in the specification, one of skill in the art would be apprised of what substantially homogeneous type II-like collagen is and the rejection should be withdrawn, accordingly.

Claims 7 and 11 are further rejected under 35 U.S.C. § 112, 2 for reciting "said salt." Applicant has removed the recitation of "said," thereby alleviating the Examiner's concerns. Applicant respectfully requests that the rejection be withdrawn.

Claims 8 and 9 are further rejected under 35 U.S.C. § 112, 2 for reciting "said salt solution." Applicant has removed the recitation of "solution," thereby alleviating the Examiner's concerns. Applicant respectfully requests that the rejection be withdrawn.

Claims 12 and 14 are further rejected under 35 U.S.C. § 112, 2. The Office Action asserts that Applicant must specify the route of administration. However, the specification provides the following.

The treatment methods of the invention comprise the step of administering an effective amount of a composition of the invention, e.g., jellyfish type II-like collagen. The compositions administered in the subject methods are administered such that the collagen contacts the lymphoid tissue of the gut (Peyer's patches, etc.) so that immune tolerance is induced. The collagen may be administered with additional pharmaceutical compounds for the treatment of arthritis, such as anti-inflammatory agents, etc.

The subject compositions may be adapted for various types of administration, e.g., topically, orally, intranasally, by injection or by inhalation. Administration of collagen may be effected by many possible methods through the use of formulations comprising the subjected compositions that are designed for oral administration, i.e., the active components are not destroyed or inactivated in the mouth, stomach, or other portions of the digestive system prior to contacting the appropriate gut lymphoid tissue.

Compositions of the invention may optionally be formulated in association with a pharmaceutically acceptable carrier. Alternatively, compositions of the invention may optionally be formulated as a pharmaceutically acceptable salt thereof, in association with a pharmaceutically acceptable carrier.

A "pharmaceutically acceptable carrier" includes solids, semi-solids or liquid diluents, or an ingestible capsule, such as tablets, gelatin capsules, drops such as nasal drops, preparations for topical application such as ointments, jellies, creams and suspensions, aerosols for inhalation, nasal spray, liposomes, etc. In most cases, the collagen will comprise between 0.05 and 99%, or between 0.1 and 99% by weight of the preparation, for example between 0.5 and 20% for preparations intended for injection and between 0.1 and 50% for preparations intended for oral administration.

For oral administration, the agent can be contained in enteric forms to survive the stomach or further coated or mixed to be released in a particular region of the GI tract by known methods. For instance, the agent can be provided with a layer of gastric juice-resistant enteric film or coating having such properties that it is not dissolved at the acidic pH in the gastric juice, thereby preventing release of the collagen until the preparation reaches the intestines. Such coatings include cellulose acetate phthalate, hydroxypropyl-methylcellulose phthalates, etc. For the purpose of oral therapeutic administration, the collagen can be incorporated with excipients and used in the form of tablets, troches, or capsules. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches, and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth, or gelatin; an excipient such as starch or lactose; a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

> Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Liquid preparations for oral application may be in the form of elixirs, syrups or suspensions. Such solutions typically contain from about 0.1% to 20% by weight of active substance, sugar and a mixture of ethanol, water glycerol, propylene glycol and optionally aroma, saccharine and/or carboxymethylcellulose as a dispersing agent. The type II-like collagen may also be formulated as a solid or liquid dietary supplement, i.e., incorporated with juice, etc.
>
> The compositions of the invention may also be prepared as a sustained release formulation. One example of a sustained release formulation is a tablet, composed of several layers of the active ingredient that are separated by coatings that dissolve slowly. Alternatively the active ingredient may be divided into particles with coatings of different thickness compressed together with a carrier substance. Alternatively, the active ingredient may be incorporated into a fat and wax substances.
>
> Administration can also be by transmucosal or transdermal means. Examples of pharmaceutical preparation for this route of administration include ointments, jellies, creams and suspensions, aerosols for inhalation, nasal spray, liposomes, etc For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives.

See the specification, page 12, line 19 to page 14, line 17. Methods and techniques for these routes of administration are known to those of skill in the art. Given the guidance in the specification, one of skill in the art would be apprised of which modes of administration are applicable. Applicant respectfully requests that the rejection be withdrawn.

Claim 16 is rejected under 35 U.S.C. § 112, 2 on the grounds that it "is improper composition claim because the claim comprises only one ingredient, namely type II-like collagen" and because "to be proper, the claim should recite more than one component or ingredient." Composition claims drawn to only one ingredient are legally permissible and regularly issue from the Patent Office. Applicant respectfully requests that the rejection be withdrawn.

Claim 16 is further rejected under 35 U.S.C. § 112, 2 on the grounds it is not clear "how much the type II-like collagen is free of natural contaminants." Applicant disagrees.

The term "substantially free of natural contaminants" has been defined on page 7, lines 24-28 of the specification to mean the following.

> By collagen that is "substantially free of natural contaminants" is intended collagen that has, at some point, been purified to be substantially homogeneous. However, collagen that is substantially free of natural contaminants may be associated with other compounds. In no case does collagen substantially free of natural contaminants encompass the collagen present in its natural source.

As discussed above with respect to the rejection of claims 1-14 and 16, one of skill in the art would understand the meaning of substantially homogeneous and the specification itself teaches that the substantially homogeneous type II-like collagen can be formulated with a pharmaceutically acceptable carrier, etc. Given the guidance provided by the specification, one of skill in the art would be appraised of the meaning of "type II-like collagen that is substantially free of natural contaminants." Thus, the claim satisfies the standard for definiteness and Applicant respectfully requests that the rejection be withdrawn.

The Rejections of the Claims Under 35 U.S.C. § 102(b) Should Be Withdrawn

Claims 15 and 16 stand rejected under 35 U.S.C. § 102(b) over U.S. Pat. No. 5,843,445 to Weiner *et al.* The rejection is grounded on the following reasoning:

> Weiner *et al.* discloses a pharmaceutical formulations comprising type II collagen for the treatment of autoimmune arthritis of animals including humans. Since the pharmaceutical formulation of type II collagen is administered by oral, enteral, or by-inhalation to humans for treatment of rheumatoid arthritis, said pharmaceutical formulation has to be free of all contaminants including natural contaminants.
>
> ...
>
> Thus, the prior art discloses the invention substantially as claimed, and as such, anticipates claims 15 and 16 as drafted.

Applicant has amended claims 15 and 16 to recite substantially homogeneous type II-like collagen from one or more species of jellyfish. Unlike Applicant's collagen, the collagen discussed in Weiner *et al.* is vertebrate type II collagen, particularly chick or bovine type II-collagen. (Chick type II collagen is utilized by Weiner *et al.* in the experimental examples.) The specification teaches that although type II-like collagen from jellyfish is similar to type II vertebrate collagen, there are differences observable by Cyanogen Bromide peptide mapping. Other differences are listed, too.

> Further, comparison of the amino acid composition of vertebrate type II collagen and type II-like jellyfish collagen show that vertebrate type II collagen has a comparatively higher proline and hydroxyproline content, while type II-like collagen has a comparatively higher hydroxylysine and lysine content.

See the specification, page 8, lines 25-30. As the Examiner is aware, peptide mapping reveals distinguishable features of the primary amino acid sequence of a polypeptide. Thus, Applicant's novel type II-like collagen from jellyfish is distinguishable from vertebrate type II collagen by (1) peptide mapping and (2) proline and hydroxyproline content. Applicant explains further differences in her Rule 132 declaration.

> The differences between invertebrate type II-like collagen and vertebrate type II collagen at the molecular level have been described above. Furthermore, although the type II-like JF collagen exhibits both significant preventive and therapeutive effects on collagen-induced arthritis (CIA), it is not antigenic like the vertebrate type II collagen: Repetitive injection of jellyfish collagen did not induce CIA in lab animals (as shown in the Example 6 of our disclosure).

See paragraph 7 of the accompanying Rule 132 declaration.

To constitute anticipatory art under Section 102, a single reference must teach every claim element. Because Weiner *et al.* does not disclose Applicant's type II-like collagen from jellyfish, the reference does not anticipate claims 15 and 16. Applicant respectfully requests that the rejection be withdrawn.

The Rejections of the Claims Under 35 U.S.C. § 103 Should Be Withdrawn

Claims 1-16 stand rejected under 35 U.S.C. § 103 over Weiner *et al.* (U.S. Pat. No. 5,843,445) in combination with Wolfinbarger (U.S. Pat. No. 5,714,582) and Neff *et al.* (U.S. Pat. No. 5,925,736). Applicant respectfully traverses.

As amended, all of the claims are drawn to compositions and methods comprising substantially homogeneous type II-like collagen from one or more species of jellyfish. As an initial matter, Applicant wishes to clarify one point raised in the rejection. The rejection asserts that on page 7 of her disclosure, "Applicant acknowledges that methods for extraction of collagen (type I-V) from jellyfish are known in the art...." To the extent the statement intends that Applicant acknowledges that methods of extracting substantially homogeneous type II-like collagen from jellyfish are in the art, Applicant traverses. Applicant emphasizes that the specification merely states that known methods for the extraction of collagen from jellyfish result in a *mixture* of collagen types. See the specification, page 8, lines 5-6 (emphasis added). It is *Applicant's* disclosure that teaches obtaining substantially homogeneous type II-like collagen from jellyfish. Applicant describes the extraction process in her accompanying declaration.

> Jellyfish contain many proteins and different types of collagens. Although methods of extraction of jellyfish collagen are known in the art, these methods extract a mixture of collagen types rather than a single type of collagen. This is the reason for controversial conclusions on collagen types in the literature (as indicated in our patent application, page 2). Our method is the first to isolate jellyfish (JF) collagens from noncollagenous proteins and then to isolate the major JF collagen, type II-like, from other collagens by salt fractionation at neutral pH. Our claimed composition comprises substantially homogeneous type II-like collagen from JF.
>
> In detail, our disclosure teaches that additional steps must be taken to achieve greater purity than the methods already in the art. For instance, our method teaches that a first step of cleaning the homogenized and freeze-dried JF tissue with water to remove water-soluble substances including salt and water-soluble proteins can be taken. In addition, our method teaches that a second step of extracting the residue with alkali solution (NaOH) to remove alkali-soluble proteins can be taken. Moreover, our method provides that a third step of further extracting the residue with 0.5 M acetic acid to remove acid-soluble non-collagenous proteins can be taken. Our protocol produces collagen as an

> insoluble residue that is solubilized by digestion with an enzyme such as pepsin as described in the methods. Over 50% of JF dry weight is pepsin-soluble collagen. Finally, our method utilizes a salt-fractionation technique to further separate the type II-like JF collagen from other proteins (contaminants) and which can be further dialyzed to remove any contaminants. If all of these steps are utilized, the purity of our type II-like JF collagen obtained after salt-fractionation (at 3.0-3.5 M NaCl, neutral pH) of the pepsin-soluble collagen is estimated to be at least 99% pure.

See paragraphs 3 and 4 of the accompanying Rule 132 declaration.

As the Examiner is aware, each collagen type is composed of a unique combination of subunits and can be identified by analytical techniques including SDS-PAGE electrophoretic mobility, amino acid composition, etc. Analysis of Applicant's novel substantially homogeneous collagen from jellyfish revealed that it is type II-like collagen. This in turn suggested that type II-like collagen from jellyfish could be utilized for immune tolerization (vertebrate type II collagen is the primary tolerizing collagen used in mammalian studies). Applicant has carried out experimental work confirming that type II-like collagen from jellyfish is a tolerizing antigen in mice (see the specification, Examples 3-6). The rejection fails to establish a prima facie case that Applicant's substantially homogeneous type II-like collagen from jellyfish or methods of using it are obvious, as will be explained below.

To establish a prima facie case of obviousness, the Office must establish that the references teach or suggest each element of the claimed subject matter. Further, the Office must establish that the references or the knowledge in the art provides the motivation for the combination of references cited and any modification thereto. The references cited in the Office Action taken individually or in combination fail meet this burden for the reasons set forth in the following pages.

The primary reference relied upon in the rejection is Weiner *et al.* This reference teaches only *vertebrate* type II collagen. As discussed with respect to the Section 102 rejection, the specification teaches that type II-like collagen from jellyfish is distinguishable on the molecular level from vertebrate type II collagen. Accordingly, Weiner *et al.* does not teach or suggest Applicant's substantially homogeneous type II-like collagen from jellyfish or methods of using the same.

Neff *et al.* fails to correct the deficiencies of the primary reference. In particular, Neff *et al.* discloses only vertebrate collagen and does not teach or suggest Applicant's substantially homogeneous type II-like collagen from jellyfish.

Wolfinbarger does not correct the deficiencies of the primary reference, either. The Office Action asserts that Wolfinbarger teaches Applicant's purification scheme, including fractionation and isolation of the 3.5 M salt fraction. Applicant's representative has diligently studied the entire reference and cannot determine where Wolfinbarger teaches that the 3.5 M fraction should be isolated from the other fractions (or even that any material precipitated at a particular molarity of salt should be isolated). For instance, Example 4 of Wolfinbarger teaches that "the sodium chloride was added in small increments and the precipitated material removed as formed by the salt precipitation. Essentially *all* of the collagen was thus precipitated by the addition of sodium chloride and was then transferred into a separate container." See Wolfinbarger, col. 10, lines 15-19 (emphasis added). Thus, Wolfinbarger teaches that all of the collagen is precipitated and transferred. It does not teach that the material precipitated at a particular molarity of salt should be selected and isolated from the other precipitated material.

In any case, Wolfinbarger fails to teach or suggest Applicant's substantially pure type II-like collagen from jellyfish. Rather, as described in the accompanying Rule 132 declaration, the method of Wolfinbarger would produce a mixture of all types of collagens and other proteins.

> The invertebrate type V collagen compositions of Wolfinbarger are conceptually and technically different from our compositions. These differences include the fact that in his proof of concept experimental Examples, Wolfinbarger starts with extraction of JF tissue with very dilute acid (0.05 N acetic acid in Example 4). This acid-soluble mixture is then filtered to collect a viscous solution, which is then precipitated by addition of solid sodium chloride to a final salt concentration of 3.5 M. The precipitated collagen is generally washed and redissolved in dilute acid (0.05 M acetic acid in Example 4), then frozen as a thin layer and freeze dried. Wolfinbarger teaches that a composition consisting essentially of invertebrate type V collagen is obtained using these techniques. Wolfinbarger fails to teach that additional steps are necessary to produce collagen with an appreciable level of purity and does not remove water-soluble, acid-soluble, or base-soluble non-collagenous proteins. For instance, in Example 4 Wolfinbarger utilizes a salt precipitation step at acidic pH rather than a fractionation procedure at neutral pH. Further, Wolfinbarger teaches the addition of salt in small increments up to roughly 3.5 M, but does not teach that the

> collagen precipitated at each molarity should be isolated as separate fractions. In particular, Wolfinbarger does not teach that the collagen precipitated in the 3.0-3.5 M salt range should be selected to the exclusion of all other fractions. It is my scientific opinion that the disclosure of Wolfinbarger fails to teach or suggest how to produce a substantially homogeneous type II-like collagen composition from JF. It is my further scientific opinion that if one followed the teachings of Wolfinbarger, a composition that includes non-collagenous proteins and various collagens that could have been separated at salt concentrations lower than 3.5 M (such as 1.8 M, 2.0 M, etc) would be obtained. Further, Wolfinbarger itself does not teach or suggest that additional steps are necessary to achieve a substantially homogeneous collagen composition.

See paragraph 6 of the accompanying Rule 132 declaration.

As Applicant states in the last sentence of paragraph 6, nothing in Wolfinbarger teaches that its collagen requires further steps for purification. To the contrary, Wolfinbarger states that by following its methods, one can obtain "by treatment with acid . . . a viscous dispersion containing native fibrillary collagen and a small amount of native collagen in solution" that "consists essentially of a invertebrate type-V collagen of the composition alpha1alpha2alpha3." See Wolfinbarger, column 6, line 63 to column 7, line 2. Thus, the reference fails to provide the motivation to carry out further purification steps. Indeed, by teaching that a composition that "consists essentially of a invertebrate type-V collagen" is obtained by following its method, Wolfinbarger teaches away from the need for carrying out additional purification steps. It is Applicant's disclosure that teaches that additional purification steps are necessary to achieve substantially homogeneous type II-like collagen from jellyfish.

To demonstrate that her jellyfish collagen is substantially homogenous and type II-like, Applicant has carried out additional experimental work (presented in Exhibit A) which she summarizes in her declaration as follows.

> Indicative of its purity, our substantially homogeneous type II-like JF collagen purified from either JF legs or JF umbrella produces essentially one major band on a 6 % SDS-polyacrylamide gel (Exhibit A, Figure 1, lanes 4 and 8). The mobility of its single major band is substantially similar to that of mammalian type II collagen, which has three identical alpha chains that migrate as a single band (Exhibit A, Figure 1, lane II). In contrast, mammalian type V collagen is composed of three different chains that migrate as three major bands (Exhibit A, Figure 1, lane V). It is my scientific opinion that under SDS-PAGE analysis, a

> crude heterogeneous preparation containing a mixture of JF collagens could produce multiple bands and therefore be mistaken for a type V-like collagen. The purity of our type II-like collagen from JF, as well as its similarity to mammalian type II collagen, has been experimentally confirmed with DSC. Like pure mammalian type II collagen (Exhibit A, Figure 2, panel C), our substantially homogeneous type II-like collagen from JF produces a single denaturation peak (Exhibit A, Figure 2, panels A & B). When compared with type I and II collagen standards, our substantially homogeneous type II-like collagen from JF produces denaturation temperatures and enthalpies similar to that of the vertebrate type II standard (Figure 3) and dissimilar from the vertebrate type I standard.

See paragraph 5 of the accompanying Rule 132 declaration.

Thus, the references cited in the rejection fail to teach or suggest that additional purification steps are required to obtain substantially homogeneous collagen from jellyfish (particularly the step of salt fractionating and selecting the 3.0-3.5 M collagen fraction) or that type II-like collagen can be obtained from jellyfish at all. Rather, it is Applicant's own disclosure that teaches obtaining substantially homogeneous type II-like collagen from jellyfish. Consequently, the rejection is based upon an improper hindsight-based analysis in which the teachings of Applicant's patent application are relied upon to cure the deficiencies of the cited art. For these reasons alone, the rejection of claims 1-16 should be withdrawn.

Applicant notes that on pages 7-8 of the Office Action, it is asserted that the methods of Weiner *et al.* would be combined with the composition of Wolfinbarger in light of Neff *et al.* to produce Applicant's methods. Applicant traverses this argument because the references fail to teach or suggest Applicant's substantially homogenous type II-like collagen from jellyfish and therefore cannot render methods of using substantially homogenous type II-like collagen from jellyfish obvious.

Moreover, the cited references fail to teach or suggest the use of *any* jellyfish collagen for the treatment of arthritis. Although Neff *et al.* is cited for the motivation to utilize Wolfinbarger's invertebrate type V collagen for inducing oral tolerance, Neff *et al.* contains only a blanket assertion that collagen types I, II, III, V, VI, IX, X, XI, XII, and XIV are useful for inducing oral tolerance. The Office Action does not support its conclusion that the art worker would find motivation in Neff *et al.* (which states that virtually all known collagen types are

useful for oral tolerization) to focus with particularity on type V collagen. Even taken at face value, Neff *et al.* can only stand for the proposition that it might be obvious to try to utilize every type of collagen for oral tolerization. However, obvious to try is not the applicable legal standard under Section 103. *See Ex parte* Levengood, 28 U.S.P.Q.2d 1300 (BPAI 1993).

In the accompanying Rule 132 declaration, Applicant explains why Neff's blanket assertion that collagen types I, II, III, V, VI, IX, X, XI, XII, and XIV are useful for inducing oral tolerance is not accepted in the art as follows.

> ...it is my scientific opinion that the Neff *et al.* patent contains a broad assertion that does not represent the view in the art at the time the present invention was made. Specifically, the skilled worker would not accept the assertion of Neff *et al.* that each of types I, II, III, V, VI, IX, X, XI, XII, and XIV would be useful for treating arthritis. In particular, the art recognizes that type V collagen is not known to induce an autoimmune response associated with arthritis. For instance, a 1981 study investigated types I-V collagen to determine which functioned as autoantigens. Unlike type II collagen, types IV and V collagen did not generate positive immune reactions with any consistency. See Trentham *et al.* (1981) *Arthritis and Rheumatism* 24:1363-1369, p. 1365, column 2. A 1998 review article identifies types II, IX, and XI collagen as the collagen types useful for treating arthritis (as well as the non-collagenous moieties HCgp-39, PG, and LP). See Trentham (1998) *Rheumatic Disease Clinics of North America* 24:525-536, p. 533. The basis for the art-consensus is explained in Malmstrom *et al.* (1996) *PNAS USA* 93:4480-4485 as follows.
>
> > Rheumatoid arthritis (RA) is an autoimmune inflammatory disease affecting peripheral joints. The main genetic association is to the major histocompatibility complex (MHC) class II region (HLA-DR), suggesting involvement of T-cell-mediated autoimmune recognition of joint-specific antigens. Known proteins within joints that can be considered joint-specific are those found in articular cartilage. These include collagens of type II (CII), type IX (CIX), and type XI (CXI), and noncollagenous proteins such as aggrecan and COMP.

See paragraph 8 of the accompanying Rule 132 declaration.

Contrary to Neff *et al.*, Weiner *et al.* expresses the accepted view of the art that that *not all* types of collagen are useful for inducing oral tolerance. In particular, it is types I, II, III, IX, and XI that have been recognized as useful for oral tolerization, with type II collagen the predominant type. See paragraph 8 of the accompanying Rule 132 declaration. Consequently,

one of skill in the art would not be motivated to modify Neff *et al.* by specifically selecting the invertebrate type V collagen of Wolfinbarger in any method of oral tolerization. Further, none of the references demonstrate that the art worker would have a reasonable chance of success of oral tolerization with Wolfinbarger's invertebrate type V collagen, nor do they provide the motivation to modify Wolfinbarger's purification method and obtain Applicant's type II-like collagen from jellyfish. The rejection of Applicant's method claims 12-14 under Section 103 should be withdrawn for the aforementioned reasons, as well.

In paragraph 9 of her declaration, Applicant summarizes her scientific opinion regarding the differences between the art cited in the rejection and her presently claimed invention.

> [I]t is my scientific opinion that the methods of Wolfinbarger would not produce our claimed composition because if one were to carry out Wolfinbarger's teachings, JF collagen may be obtained, but it would be of low purity and heterogeneous in nature. Further, it is my scientific opinion that the methods of Weiner *et al.* would fail to motivate one in this field to combine its teachings with the invertebrate type V collagen of Wolfinbarger because Weiner *et al.* expresses the accepted scientific view that type V collagen is not useful in methods of oral tolerization for the treatment of arthritis. Moreover, it is my scientific opinion that those in this field would not accept the blanket assertion of Neff *et al.* that all forms of collagen could be used for the treatment of arthritis and, consequently, would not be motivated to combine its teachings with the invertebrate type V collagen of Wolfinbarger. For the above reasons, based on my education and scientific experience, I believe that one working in this field would not arrive at our claimed compositions or methods given the references by Weiner *et al.*, Wolfinbarger, and Neff *et al.* Based on my education and scientific experience, I further do not believe the references themselves or the state-of-art would motivate one working in this field to combine or modify these references in the way asserted in the rejection.

Thus, the rejection fails to establish a *prima facie* case that Applicant's substantially homogeneous type II-like collagen or its use for inducing oral tolerance would be obvious because of multiple deficiencies including the following.

- There is no teaching or suggestion in the cited art of type II-like collagen from jellyfish.
- There is no teaching or suggestion in the cited art of how to obtain substantially homogeneous type II-like collagen.

- The motivation to combine or modify the references in the fashion asserted in the Office Action has not been established: The secondary reference to Weiner *et al.*, as well as the knowledge of those of skill in the art, teaches away from utilizing type V collagen for oral tolerization. Thus, both the art in general and the secondary reference to Weiner *et al.* teach away from the combination of Wolfinbarger with any cited reference.
- The cited art fails to establish that the person of ordinary skill would have a reasonable chance of success of arriving at Applicant's claimed invention.

Even if the assertions within the Office Action were sufficient to establish a *prima facie* case, the accompanying Rule 132 evidence would rebut it. Accordingly, Applicant respectfully requests that the rejection of claims 1-16 be withdrawn.

CONCLUSION

In view of the aforementioned amendments and remarks, Applicant respectfully submits that the objection to the specification and the rejections of the claims under 35 U.S. C. §§ 102, 103, and 112, paragraph 1 are overcome. Accordingly, Applicant submits that this application is now in condition for allowance. Early notice to this effect is solicited. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of

Attorney's Docket No. 35721/239475(5721-17) PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Hsieh	Confirmation No.:	6335
Appl. No.:	10/007,716	Group Art Unit:	1653
Filed:	November 13, 2001	Examiner:	Abdel A. Mohamed
For:	USE OF JELLYFISH COLLAGEN (TYPE II) IN THE TREATMENT OF RHEUMATOID ARTHRITIS		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RULE 37 C.F.R. § 1.132 DECLARATION
of Yun-Hwa Peggy Hsieh

I, Yun-Hwa Peggy Hsieh, do hereby declare and say as follows:

1. I, Yun-Hwa Peggy Hseigh, am skilled in the art of the field of the invention. I have Ph.D. in Food Science from Florida State University. I have a Master of Science degree in Animal Sciences from Purdue University, West Lafayett, Indiana. I have a Bachelor of Science degree in Nutrition and Food Sciences Fu-Jen University, Taipei, Taiwan. I have been employed as a professor by Florida State University , Tallahassee, Florida from 2003 to present. Prior to Florida State University, I was employed by Auburn University, Auburn, Alabama from 1993 to 2002.

2. I have read and understood the Office Action in the above case dated 26 February 2004. I have also read and understood references cited and discussed in this case, including Weiner *et al.* (U.S. Pat. No. 5,843,445) and Wolfinbarger (U.S. Pat. No. 5,714,587) and Neff *et al.* (U.S. Pat. No. 5,925,736). Our claimed compositions and methods are conceptually and technically different from those of the references cited in the official action.

3. Jellyfish contain many proteins and different types of collagens. Although methods of extraction of jellyfish collagen are known in the art, these methods extract a mixture

of collagen types rather than a single type of collagen. This is the reason for controversial conclusions on collagen types in the literature (as indicated in our patent application, page 2). Our method is the first to isolate jellyfish (JF) collagens from noncollagenous proteins and then to isolate the major JF collagen, type II-like, from other collagens by salt fractionation at neutral pH. Our claimed composition comprises substantially homogeneous type II-like collagen from JF.

4. In detail, our disclosure teaches that additional steps must be taken to achieve greater purity than the methods already in the art. For instance, our method teaches that a first step of cleaning the homogenized and freeze-dried JF tissue with water to remove water-soluble substances including salt and water-soluble proteins can be taken. In addition, our method teaches that a second step of extracting the residue with alkali solution (NaOH) to remove alkali-soluble proteins can be taken. Moreover, our method provides that a third step of further extracting the residue with 0.5 M acetic acid to remove acid-soluble non-collagenous proteins can be taken. Our protocol produces collagen as an insoluble residue that is solubilized by digestion with an enzyme such as pepsin as described in the methods. Over 50% of JF dry weight is pepsin-soluble collagen. Finally, our method utilizes a salt-fractionation technique to further separate the type II-like JF collagen from other proteins (contaminants) and which can be further dialyzed to remove any contaminants. If all of these steps are utilized, the purity of our type II-like JF collagen obtained after salt-fractionation (at 3.0-3.5 M NaCl, neutral pH) of the pepsin-soluble collagen is estimated to be at least 99% pure.

5. We have analyzed our substantially homogeneous type II-like JF collagen by SDS-PAGE and micro differential scanning calorimetry (DSC) (see Figures 1-3 of Exhibit A, attached). Indicative of its purity, our substantially homogeneous type II-like JF collagen purified from either JF oral arms (legs) or JF umbrella produces essentially one major band on a 6 % SDS-polyacrylamide gel (Exhibit A, Figure 1, lanes 4 and 8). The mobility of its single major band is substantially similar to that of mammalian type II collagen, which has three identical alpha chains that migrate as a single band (Exhibit A, Figure 1, lane II). In contrast, mammalian type V collagen is composed of three different chains that migrate as three major

bands (Exhibit A, Figure 1, lane V). It is my scientific opinion that under SDS-PAGE analysis, a crude heterogeneous preparation containing a mixture of JF collagens could produce multiple bands and therefore be mistaken for a type V-like collagen. The purity of our type II-like collagen from JF, as well as its similarity to mammalian type II collagen, has been experimentally confirmed with DSC. Like pure mammalian type II collagen (Exhibit A, Figure 2, panel C), our substantially homogeneous type II-like collagen from JF produces a single denaturation peak (Exhibit A, Figure 2, panels A & B). When compared with type I and II collagen standards, our substantially homogeneous type II-like collagen from JF produces denaturation temperatures and enthalpies similar to that of the vertebrate type II standard (Exhibit A, Table 1) and dissimilar from the vertebrate type I standard. However, although our substantially homogeneous type II-like collagen is very similar to vertebrate type II collagen, some differences exist at the molecular level. For instance, CM-52 Chromatographic separation of the jellyfish type II-like collagen produces two major peaks, as described in Example 1 of our disclosure. Further, comparison of the amino acid composition of vertebrate type II collagen and type II-like jellyfish collagen show that vertebrate type II collagen has a comparatively higher proline and hydroxyproline content, while type II-like collagen has a comparatively higher hydroxylysine and lysine content. Because of these characteristics, our substantially homogenous type II-like JF collagen differs from the collagen of the cited references, as is described in the following paragraphs.

6. The invertebrate type V collagen compositions of Wolfinbarger are conceptually and technically different from our compositions. These differences include the fact that in his proof of concept experimental Examples, Wolfinbarger starts with extraction of JF tissue with very dilute acid (0.05 N acetic acid in Example 4). This acid-soluble mixture is then filtered to collect a viscous solution, which is then precipitated by addition of solid sodium chloride to a final salt concentration of 3.5 M. The precipitated collagen is generally washed and redissolved in dilute acid (0.05 M acetic acid in Example 4), then frozen as a thin layer and freeze dried. Wolfinbarger teaches that a composition consisting essentially of invertebrate type V collagen is obtained using these techniques. Wolfinbarger fails to teach that additional steps are necessary to produce collagen with an appreciable level of purity and does not remove water-soluble, acid-

soluble, or base-soluble non-collagenous proteins. For instance, in Example 4 Wolfinbarger utilizes a salt precipitation step at acidic pH rather than a fractionation procedure at neutral pH. Further, Wolfinbarger teaches the addition of salt in small increments up to roughly 3.5 M, but does not teach that the collagen precipitated at each molarity should be isolated as separate fractions. In particular, Wolfinbarger does not teach that the collagen precipitated in the 3.0-3.5 M salt range should be selected to the exclusion of all other fractions. It is my scientific opinion that the disclosure of Wolfinbarger fails to teach or suggest how to produce a substantially homogeneous type II-like collagen composition from JF. It is my further scientific opinion that if one followed the teachings of Wolfinbarger, a composition that includes non-collagenous proteins and various collagens that could have been separated at salt concentrations lower than 3.5 M (such as 1.8 M, 2.0 M, etc) would be obtained. Further, Wolfinbarger itself does not teach or suggest that additional steps are necessary to achieve a substantially homogeneous collagen composition.

7. The compositions and methods of Weiner *et al.* are conceptually and technically different from our compositions and methods. These differences include the fact that our claimed composition comprises type-II-like collagen from invertebrate JF. Weiner *et al.* discloses and utilizes type II collagen from vertebrate sources, specifically chicken and cow. The differences between invertebrate type II-like collagen and vertebrate type II collagen at the molecular level have been described above. Furthermore, although the type II-like JF collagen exhibits both significant preventive and therapeutive effects on collagen-induced arthritis (CIA), it is not antigenic like the vertebrate type II collagen: Repetitive injection of jellyfish collagen did not induce CIA in lab animals (as shown in the Example 6 of our disclosure).

8. The compositions and methods of Neff *et al.* are conceptually and technically different from our compositions and methods because Neff *et al.* utilize type II collagen from vertebrate sources. Further, it is my scientific opinion that the Neff *et al.* patent contains a broad assertion that does not represent the view in the art at the time the present invention was made. Specifically, the skilled worker would not accept the assertion of Neff *et al.* that each of types I, II, III, V, VI, IX, X, XI, XII, and XIV would be useful for treating arthritis. In particular, the art

recognizes that type V collagen is not known to induce an autoimmune response associated with arthritis. For instance, a 1981 study investigated types I-V collagen to determine which functioned as autoantigens. Unlike type II collagen, types IV and V collagen did not generate positive immune reactions with any consistency. See Trentham *et al.* (1981) *Arthritis and Rheumatism* 24:1363-1369, p. 1365, column 2. A 1998 review article identifies types II, IX, and XI collagen as the collagen types useful for treating arthritis (as well as the non-collagenous moieties HCgp-39, PG, and LP). See Trentham (1998) *Rheumatic Disease Clinics of North America* 24:525-536, p. 533. The basis for the art-consensus is explained in Malmstrom *et al.* (1996) *PNAS USA* 93:4480-4485 as follows.

> Rheumatoid arthritis (RA) is an autoimmune inflammatory disease affecting peripheral joints. The main genetic association is to the major histocompatibility complex (MHC) class II region (HLA-DR), suggesting involvement of T-cell-mediated autoimmune recognition of joint-specific antigens. Known proteins within joints that can be considered joint-specific are those found in articular cartilage. These include collagens of type II (CII), type IX (CIX), and type XI (CXI), and noncollagenous proteins such as aggrecan and COMP.

See page 4480. The blanket assertion of Neff *et al.* is even contrary to the other art on which the rejection is based: Weiner *et al.* notes the existence of type V collagen, but states the following.

> The present invention is based on the discovery and confirmation that oral, enteral or by-inhalation administration of type I, type II or type III collagen or its biologically active peptide fragments in small amounts is a particularly effective means of suppressing T-cell-mediated or T-cell dependent autoimmune and particularly rheumatoid arthritis in humans. Thus, as demonstrated below, the simple method of administration, orally, enterally or by inhalation, of at least one of type I, type II or type III collagen or active fragments or analogs of at least one of them, as taught by the invention, is an effective treatment to suppress the development of arthritis. Furthermore, the compositions and method of the invention do not have the drawbacks described above and associated with prior art therapeutic or palliative agents and techniques.
>
> Oral, enteral or by-inhalation induced tolerance is dose-dependent over a broad range of oral, enteral and inhalant dosages. However, there are minimum and maximum effective dosages. As is understood by one skilled in the art, this means that suppression of both clinical and histological symptoms of arthritis occurs within a specific dosage range which varies as a function of the type of the collagen protein administered, whether it is whole protein or discrete peptide

> fragment(s) or analog(s), as well as the solubility and purity of the peptides or polypeptides. Moreover, the age, sex and physical condition of the patient, as well as other concurrent treatments being administered also have a bearing on the effective dosage of collagen protein for treatment. Consequently, adjustment and refinement of the dosages used and administration schedules must be determined based on these factors, and may need to be determined experimentally. Such determinations, however, require no more than routine experimentation. Type II collagen and its fragments and analogs are most preferred.
>
> Generally, the preferred way to accomplish suppression of the immune responses against the human body's collagen in arthritis is the administration, orally, enterally or by inhalation of purified or highly purified water-soluble whole type I, type II or type III collagen protein or its biologically active peptide fragment(s)....

It is particularly notable that Neff *et al.* presents data for only types I-III collagen. In my scientific opinion, the skilled person would not accept the teaching of Neff *et al.* that all forms of collagen are useful for treating arthritis.

9. In summary, it is my scientific opinion that the methods of Wolfinbarger would not produce our claimed composition because firstly, the type V collagen obtained by using Wolfinbarger's method is in the acetic acid-soluble portion, while the type II-like collagen isolated by our method is in the insoluble residue portion after acetic acid extraction, thus they are exclusively different; and secondly, if one were to carry out Wolfinbarger's teachings, JF collagen may be obtained, but it would be of low purity and heterogeneous in nature. Further, it is my scientific opinion that the methods of Weiner *et al.* would fail to motivate one in this field to combine its teachings with the invertebrate type V collagen of Wolfinbarger because Weiner *et al.* expresses the accepted scientific view that type V collagen is not useful in methods of oral tolerization for the treatment of arthritis. Moreover, it is my scientific opinion that those in this field would not accept the blanket assertion of Neff *et al.* that all forms of collagen could be used for the treatment of arthritis and, consequently, would not be motivated to combine its teachings with the invertebrate type V collagen of Wolfinbarger. For the above reasons, based on my education and scientific experience, I believe that one working in this field would not arrive at our claimed compositions or methods given the references

by Weiner *et al.*, Wolfinbarger, and Neff *et al.* Based on my education and scientific experience, I further do not believe the references themselves or the state-of-art would motivate one working in this field to combine or modify these references in the way asserted in the rejection.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Peggy Hsieh
Yun-Hwa Peggy Hsieh

July 22, 2004
Date

AUTOIMMUNITY TO COLLAGEN

A Shared Feature of Psoriatic and Rheumatoid Arthritis

DAVID E. TRENTHAM, GARY M. KAMMER, W. JOSEPH McCUNE, and JOHN R. DAVID

Thirty-one patients with seronegative psoriatic arthritis, 27 patients with rheumatoid arthritis, 10 with psoriasis alone, and 20 normal volunteers were studied for autoimmunity to human collagens. Cellular and humoral responses were frequently observed in the 2 groups with arthritis, but the subjects without arthritis did not display reactivity to native collagens. These data demonstrate that collagens function as autoantigens in both psoriatic and rheumatoid arthritis.

Psoriatic arthritis is an inflammatory arthritis of unknown etiology that occurs in approximately 5 to 7% of persons with psoriasis (1). The disorder is characterized by an asymmetric distribution of arthritis at onset of disease, the frequent coexistence of dystrophy of the nails and spondylitis, an increased prevalence of psoriasis and psoriatic arthritis in first-degree relatives, and the absence of IgM rheumatoid factors (1,2). These features have prompted most observers to regard psoriatic arthritis as a distinct clinical entity, although the polyarthritis in some of these patients is indistinguishable from rheumatoid arthritis (RA) (1,2). Certain immunologic findings have been reported in both psoriatic arthritis and RA, such as the presence of autoantibodies (1,3), circulating immune complexes (4,5), and depressed proliferative responses of lymphocytes to mitogens in vitro (6,7).

The observation that sensitization to native type II collagen can induce arthritis in rats (8–14) or mice (15), coupled with demonstrations of humoral (16–23) and cellular (22,24–28) responses to collagens in patients with RA, have focused attention on the possible role of collagen autoimmunity in the pathogenesis of chronic arthritic diseases. Distinct collagen types provide structural support for various mammalian tissues. Types I and III collagens are widely distributed in dermal and organ parenchyma; type II collagen appears to be restricted to cartilaginous and ocular tissues; and types IV and V collagens are recently isolated species accounting for a small proportion of the collagen content of some connective tissues (29). In this article, we report that some of these collagens function as cellular and humoral autoantigens in psoriatic arthritis as well as in RA.

MATERIALS AND METHODS

Patients

Psoriatic arthritis. We studied 31 patients with psoriatic arthritis (mean age, 54 years; range, 28–72) who were part of a larger population that we recently analyzed (1).

A preliminary account of part of this work was presented at the Thirty-seventh Annual Meeting of The American Federation for Clinical Research, May 10–12, 1980, Washington, DC and was published in Clinical Research 28:350A, 1980.

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Fifty-five percent were men. According to our classification system for psoriatic arthritis (1), 17 of the patients had asymmetric, oligoarticular arthritis (group I); 5 had symmetric arthritis affecting any pair of joints, including the distal interphalangeal and/or proximal interphalangeal joints of the hands and feet (group II); and 9 had spondylarthritis consisting of clinical and radiographic evidence of sarcoiliitis or ankylosing spondylitis (group III). All of the patients in group III had peripheral arthritis; it was characterized as a group I pattern in 7 and was typical of the group II distribution in the remaining 2.

Onychodystrophy and distal interphalangeal involvement of the fingers were present in 5 patients with psoriatic arthritis, and arthritis mutilans was documented radiographically in 3. The mean duration of psoriasis was 15 years (range, 2–37 years), and the mean duration of arthritic symptoms was 14 years (range, 2–35 years). At the time of study, all patients had skin lesions typical of psoriasis, and 90% had swelling of at least 1 joint.

None of the sera from psoriatic arthritis patients contained rheumatoid factor by latex fixation, although 1 patient had been seropositive in the past. An immunofluorescence technique detected antinuclear antibodies in 1 patient with psoriatic arthritis. All of the patients with psoriatic arthritis had been typed previously for HLA antigens at the A and B loci (1). The mean erythrocyte sedimentation rate (by the Westergren method) was 46 mm/hour, and the mean hematocrit was 39%. In the 31 patients, therapy consisted of the following: salicylates in 14; nonsteroidal antiinflammatory agents in 15; gold salts in 10; hydroxychloroquine in 5; prednisone (doses of 10 mg or less a day) in 2; 6-mercaptopurine in 1; and photochemotherapy (PUVA) in 1 (30).

Control groups. Control groups consisted of 27 patients with definite or classic RA by the criteria of the American Rheumatism Association (ARA) (mean age, 58 years; range, 32–85), 10 patients with psoriatic skin lesions but no history or clinical evidence of arthritis (mean age, 51 years; range, 33–74), and 20 normal subjects (mean age, 39 years; range, 21–75). Of the patients with RA, 82% were women. In this group, the mean duration of arthritic symptoms was 12 years (range, 6 months–50 years); 82% were judged to be functional class III or IV by the criteria of the ARA, and 74% possessed radiographic evidence of bone erosions. Rheumatoid factor and antinuclear antibodies were present, respectively, in the sera of 74% (titer range, 1:640 to 1:40, 960) and 18% (titer range, trace to 1:640) of the patients with RA. In the group with psoriasis alone, the mean duration of skin involvement was 13 years (range, 2–27 years).

Collagens. Previously prepared (8) pepsin-solubilized native human collagen (types I, II, and III) and their denatured α chains, along with pepsin-modified native type II collagen from lathyritic chick sterna, were used as test antigens. Pepsin-solubilized native type I collagen from human bone was the gift of Dr. Stephen Krane, and native types IV and V collagens, solubilized from human placentas by limited pepsin-digestion, were provided by Dr. Robert Trelstad.

Cellular sensitivity. After receiving informed consent, we obtained 120 ml of blood from each subject. All assays were performed on coded specimens. To quantify cellular sensitivity to collagen in vitro, antigen-induced leukocyte inhibitory factor production by blood mononuclear cells was measured by an indirect technique (22). Almost all assays included cells from arthritic and nonarthritic subjects cultured with the appropriate concentrations of particulate human types I, II, and III collagens, as well as the control antigen, streptokinase–streptodornase (22). Aliquot portions of the collagen solutions were freeze-dried in the culture tubes prior to the addition of culture medium and mononuclear cells (22,28) to insure that the collagen was in the native state at the onset of culture.

Leukocyte inhibitory factor activity was calculated by the following formula:

$$\% \text{ inhibition} = 1.0 - \frac{\text{Area of migration in the supernatant from cultures with antigen}}{\text{Area of migration in the supernatant from cultures without antigen}} \times 100$$

Migration inhibition responses exceeding the mean +2 standard deviations of the inhibitory responses to each collagen observed in the 20 normal subjects were considered to represent significant activity (22). Twenty percent or greater migration inhibition has been previously calculated to represent significant activity to streptokinase-streptodornase (31). A negative value for percent migration inhibition responses indicates that the area of migration occurring in the antigen-derived supernatant has exceeded the area obtained with the control supernatant. Technical factors occasionally affect the migration of the indicator cells from the capillary tubes and partially account for the variability inherent in this assay.

Antibody assays. Serum samples were examined for antibodies to native types I, II, and III collagens as well as to their α chains by passive microhemagglutination (9). A solid-phase radioimmunoassay was also used to detect antibodies to native type II collagen (32). Absorption experiments (22) in which reference antisera from rats (9) and sera from patients with RA were used verified that this radioimmunoassay detected antibodies specific for native type II collagen. We used pepsin-solubilized chick type II collagen in the radioimmunoassay to conserve human collagen after finding that antibodies to type II collagen in sera from patients with RA exhibited extensive cross reactivity with our chick and human preparations. To derive values that represent significant antibody levels to type II collagen by radioimmunoassay, the bindings observed in sera from 23 normal subjects were recorded and used to calculate the random distribution. Binding exceeding the mean +2 standard deviations of these control values was then considered to represent a significant antibody response to type II collagen.

Statistical analysis. Continuous variables were analyzed in terms of their group means (Student's *t*-test), and dichotomous variables by their proportionate group frequencies (chi square test).

RESULTS

Cellular sensitivities to collagens. The individual values for leukocyte inhibitory factor activity induced

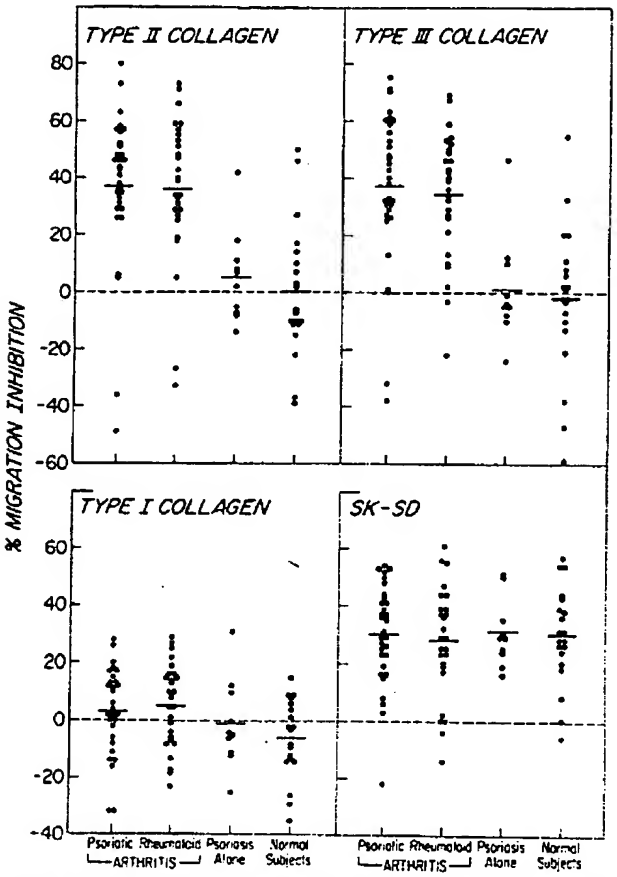

Figure 1. Leukocyte inhibitory factor responses to native human types II, III, and I collagens and the control antigen, streptokinase-streptodornase (SK-SD), depicted as percent migration inhibition, by cells from 31 patients with psoriatic arthritis, 27 patients with RA, 10 patients with psoriasis alone, and 20 normal subjects. On rare occasions, insufficient cell yields precluded cultures with type I collagen or SK-SD (horizontal lines indicate group means).

by native human types I, II, and III collagens and streptokinase-streptodornase in blood mononuclear cells from subjects in the study groups are plotted in Figure 1. Cells from 31 patients with psoriatic arthritis and 27 patients with RA generated comparable activities when cultured in the presence of type II collagen (mean ± SEM percent leukocyte inhibitory factor production to type II collagen of 37 ± 5 in the psoriatic arthritis group and 36 ± 5 in the RA group versus 0 ± 5 in the normal group, $P < 0.0001$ for both responses).

Cultures with type III collagen yielded similar results (mean percent leukocyte inhibitory factor production to type III collagen of 37 ± 5 in the psoriatic arthritis group and 34 ± 4 in the RA group versus −2 ± 6 in the normal group, $P < 0.0001$ for both responses). Cells from 48% of the psoriatic arthritis patients and 37% of the RA patients exhibited a significant response to type II collagen. Significant responses to type III collagen were observed in cells from 36% and 26% of the patients with psoriatic arthritis and RA, respectively. Cultures with type I collagen, prepared either from skin (Figure 1) or bone (data not shown) did not stimulate production of appreciable levels of leukocyte inhibitory factor by cells from either group. Cells from patients with psoriasis alone did not produce leukocyte inhibitory factor in response to these collagens. Cells from 75–80% of the subjects in all 4 groups reacted to streptokinase-streptodornase. In the arthritic groups, responses to collagens and streptokinase-streptodornase were unrelated. Thus, the absence of leukocyte inhibitory factor production to streptokinase-streptodornase did not indicate that a generalized state of impaired cellular responsiveness existed in individual arthritic patients.

To define further the ability of the various collagens to stimulate leukocyte inhibitory factor production in vitro, cells from 11 patients with RA were also cultured with native human types IV and V collagens. As with type I collagen, positive responses to these collagens did not occur with any consistency (Figure 2).

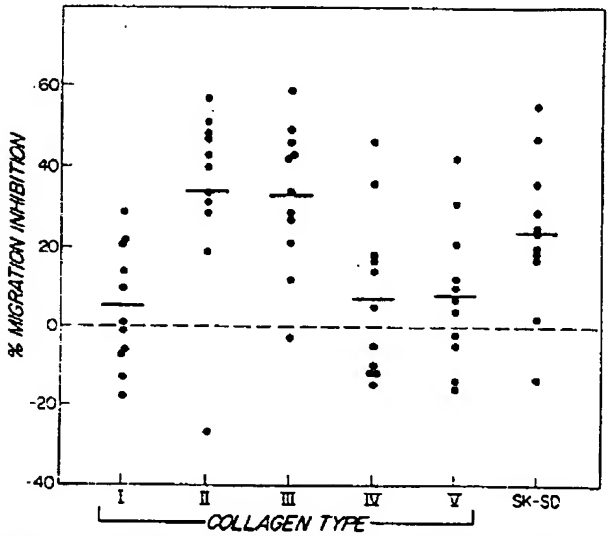

Figure 2. Inability of native human types I, IV, and V collagens to consistently induce leukocyte inhibitory factor production by cells from 11 patients with RA. Their responses to types II and III collagens as well as streptokinase-streptodornase (SK-SD) are similar to those observed in the RA group as a whole (horizontal lines indicate group means).

Table 1. Prevalence of hemagglutinating antibodies to human native collagens and their denatured α chains in sera from 31 patients with psoriatic arthritis, 27 patients with RA, and 10 patients with psoriasis alone*

	Percent of sera positive†		
	Psoriatic arthritis	Rheumatoid arthritis	Psoriasis alone
Collagen			
Type I	26	30	0
Type II	74	67	0
Type III	48	48	0
Chain			
α1 (I)	16	18	10
α2	26	37	10
α1 (II)	64	56	0
α1(III)	32	52	10

* No antibodies to collagens or α chains were found in the sera of 20 normal subjects.
† Serial $-\log_2$ titers of ≥ 2 dilutions (1:4) considered positive.

Autoantibodies to collagens. We found a comparable prevalence of type-specific (20,22) autoantibodies to native and denatured human collagens by passive hemagglutination in sera from patients with either psoriatic arthritis or RA (Table 1). Titers ranged from 2 to 6 $-\log_2$ dilutions. Although we did not detect antibodies to these preparations in normal subjects in either this study or in our previous survey (22), serum from 1 patient with psoriasis alone contained hemagglutinating antibodies to the α chains of types I and III collagens. Significantly elevated levels of antibodies to native type II collagen were also observed by the solid-phase radioimmunoassay in sera from 37% of the patients with both psoriatic arthritis and RA (Figure 3).

Presence of autoreactivity to collagens in all subgroups of psoriatic arthritis. We frequently found cellular and humoral responses to collagens in all psoriatic arthritis patient groups, including those with arthritis mutilans or whose arthritis consisted solely of distal interphalangeal involvement. Responses were noted in spondylitic and nonspondylitic patients with psoriatic arthritis, although patients with spondylitis but no peripheral arthritis were not included in this study. There was no association between humoral or cellular responses to collagens and alleles of the HLA-A or B loci in the patients with psoriatic arthritis. Moreover, these reactivities to collagens did not correlate with any clinical, laboratory, radiographic, or therapeutic feature in the patients with either psoriatic arthritis or RA.

DISCUSSION

Patients with psoriatic arthritis who are seronegative for rheumatoid factor by latex fixation exhibit cellular and humoral reactivities to human collagens with a frequency comparable to that observed in patients with RA. The presence of these autoimmune responses was not related to a symmetrical distribution of synovitis (group II) in patients with psoriatic arthritis, since patients with an asymmetric (group I) or exclusively distal interphalangeal joint involvement were also frequently found to be sensitized to collagen. Thus, autoimmunity to collagens was representative of the entire population with psoriatic arthritis and was not merely contributed by a particular group who might have the coincidental occurrence of psoriasis and RA (33).

This study provides additional evidence that

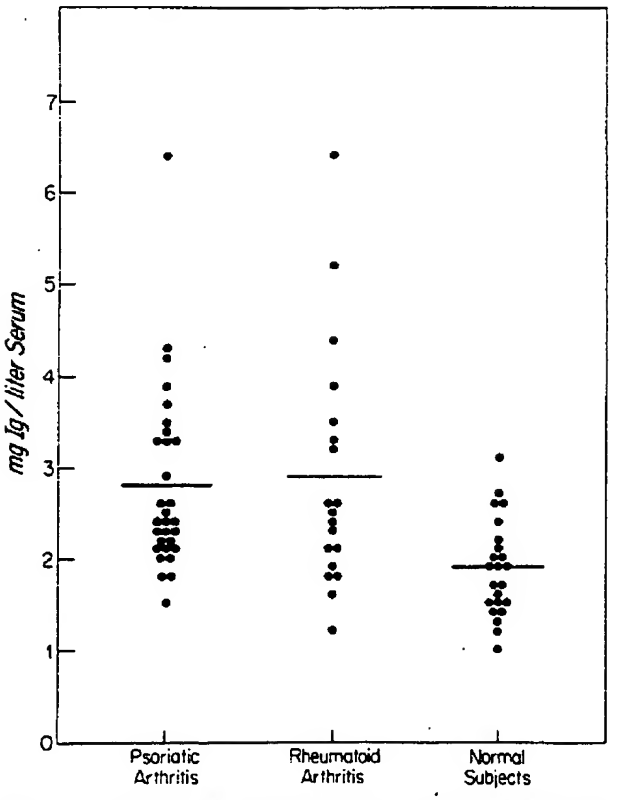

Figure 3. Antibody titers to native type II collagen, expressed as mg immunoglobulin (Ig)/liter serum (32), by solid-phase radioimmunoassay performed on sera from 30 patients with psoriatic arthritis, 19 patients with RA, and 23 normal subjects. The mean binding (horizontal lines) was significantly ($P < 0.001$) higher in both the psoriatic arthritic and RA sera than in the normal sera.

psoriatic arthritis and RA share certain immunologic features. Recent immunogenetic studies indicate that a common link between the 2 diseases may be the HLA–DRw4 locus, since the frequency of this allele has been reported to be increased in psoriatic arthritis (34) as well as in rheumatoid arthritis (35,36). Human immune response (Ir) genes appear to be localized near the D locus (37,38). Although Smolen et al (25) have reported that thymidine incorporation responses to denatured type I collagen by cells from patients with RA are not associated with the presence of HLA–DRw4, we have initiated a study to determine whether the capacity to respond immunologically to native type II collagen is related to D locus alloantigens.

Our frequent identification of antibodies to native and denatured interstitial collagens in psoriatic arthritis contrasts with a recent report which failed to detect any antibodies to native bovine types I and II collagens in the sera of 14 patients with seronegative psoriatic arthritis (23). This study utilized solid-phase radioimmunoassay. Use of heterologous collagens by these investigators is unlikely to account for these discrepant results, since antibodies to native human type II collagen exhibited extensive cross-reactivity with chick type II collagen in our solid-phase radioimmunoassay. Moreover, the prevalence of antibodies to native type II collagen in their patients with RA (42%) approximates our results (37%). As noted by these authors (23), their psoriatic patients had relatively mild arthritis, in contrast to our psoriatic arthritis group. At present, we consider this patient population difference to be the most plausible explanation for the variant findings.

It is possible that the acquisition of collagen reactivity in patients with psoriatic arthritis or RA occurs at a later stage when immunocompetent cells encounter collagen which has been exposed (39) or altered in these diseases. A variety of diseases, other than RA and psoriatic arthritis, that involve inflammation of articular, dermal, or parenchymatous structures are also associated with immunologic responses to collagen. These include the demonstrations of antibodies to type I collagen in the sera of patients with emphysema (40), leprosy (41), and from our preliminary observation in this study, psoriasis. Antibodies to type II collagen have also been found in relapsing polychondritis (42), ankylosing spondylitis (23), and systemic lupus erythematosus (D. Trentham, unpublished data). Cellular sensitivity to type I collagen was reported in patients with pulmonary fibrosis (43) and systemic sclerosis (44). In contrast with these inflammatory diseases, we found neither antibodies nor cellular reactivity to collagens in 20 patients with osteoarthritis (22). This expanded disease spectrum in which autoimmunity to collagen is a shared feature may indicate that this reactivity is a late manifestation that results from inflammatory destruction of articular or extraarticular components.

Alternatively, the emergence of a clone of lymphocytes that exhibits autoreactivity to collagen may be an early event in the primary pathogenesis of psoriatic arthritis and RA. The several clinical features which appear to distinguish psoriatic arthritis from RA (1,2) do not invalidate the hypothesis that autoimmune reactivities specific for collagens are related to the pathogenesis of both diseases. For example, there is evidence that immune responses induced by type II collagen can be associated experimentally with more than 1 form of tissue inflammation (45). While the prototypic lesion produced by injection of rodents with native type II collagen emulsified in incomplete Freund's adjuvant is a proliferative synovitis (8), on occasion an identical injection protocol results in auricular chondritis as well as arthritis (46). Thus, it is conceivable that autoreactivities specific for the same host component could contribute to morphologically dissimilar inflammatory states. An analogous situation could apply to psoriatic arthritis and RA as well as to relapsing polychondritis (42). Genetic factors may, at least partially, determine the clinical manifestations produced by autoimmune responses to collagen. The presently available data do not distinguish whether autoreactivities to collagen play a causal or secondary role in human diseases. By either mechanism, however, lymphocyte-collagen interactions could release humoral or cellular products that are capable of contributing to the connective tissue inflammation characteristic of all of these diseases.

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EMERGING THERAPIES FOR
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ORAL TOLERIZATION AS A TREATMENT OF RHEUMATOID ARTHRITIS

David E. Trentham, MD

Considerable evidence indicates that rheumatoid arthritis (RA) is a disease propagated by T-cell cytokines released in response to specific autoantigenic stimuli sequestered within joint tissue.[26, 28] Monocyte products, i.e., monokines such as tumor necrosis factor-α (TNF-α) also appear to be important but their generation may be a by-product secondarily governed by the fundamental T-cell autoreactivity. In this scenario, the major cartilage collagen species, type II collagen, in a native triple helical state, continues to be a plausible candidate for stimulating T cells in this disorder. If this pathway is operative in RA, designing a mechanism whereby antigen specific immunosuppression could be achieved would provide a new way to attenuate rheumatoid synovitis.

Oral administration of type II collagen has accomplished this feat in a number of animal models of RA.[25, 32, 39, 40] In animals, this phenomenon, termed oral tolerization, is strikingly dose-dependent in that only a narrow low-dose range produces protection. Preliminary testing in patients with RA has revealed a similar dose-related therapeutic profile for animal derived, native type II collagen.[2, 29, 30, 33] In all studies, a unique safety profile has been evident. Further analysis, perhaps incorporating human collagen made by recombinant technology, has the potential to provide a major advance in the treatment of RA.

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CANDIDATE AUTOANTIGENS IN RHEUMATOID ARTHRITIS

More than 2 decades ago, native type II collagen was serendipitously discovered to be a brisk arthritogen when used in an intradermal immunization protocol in rodents.[34] Even homologous protein emulsified in an oil vehicle devoid of other additives induced a morphologic counterpart of RA. Subsequent study has identified autoimmune responses to type II collagen in many patients with RA.[2, 6, 18, 21, 22, 31, 33] Although the frequency of humoral and cellular responses has varied in different studies, autoantibodies to type II collagen have been detected in both early and late stages of RA.[2, 6, 21] In general, the overall incidence of serum IgG antibodies is between 20% to 30% of patients. No firm connections with HLA-DR haplotype, disease manifestations, or treatment patterns have been apparent.

In RA, antibodies to two other cartilage-specific collagens have been found, mainly in association with responses to type II collagen.[21, 22] These so-called minor collagens (i.e., types IX and XI) comprise less than 5% of the matrix collagen content of hyaline cartilage.[4, 9, 27] Type II collagen is comprised of three identical α1 (II) chains. Type IX collagen is a nonfibrillar collagen and is shorter in length than type II or XI collagens. It is made up of three genetically distinct triple-helical domains interspersed with noncollagenous regions. Because it is covalently cross-linked to type II collagen, type IX collagen may provide a link between the dominant type II collagen fibers and even help adherence to proteoglycans.[9, 27] Type XI collagen is comprised of distinct triple helical α1 (XI), α2(XI), and α3(XI) chains. The α3 (XI) chain is biochemically similar to the α1(II) chain of type II collagen. It is not surprising that both type II and XI collagens are immunogenic and arthritogenic in rats, and that their respective antibodies display some cross-reactivity.[3, 21, 22, 23]

In RA sera, there are highly heterogeneous antibody responses to a variety of epitopes on native and denatured types II, IX, and XI collagen.[2, 21, 22, 31] Antibodies to the other two major interstitial collagens, types I and III may be present as well.[22] A recent survey has indicated shifts within patients in collagen autoantibody profiles as a function of time.[21] It is unclear whether these alterations are attributable to immunogenetic factors, type of collagen release from cartilage at different stages of disease, or treatment influences.

A multitude of other cartilage constituents exists, each of which could play a singular or contributory role in the autoimmune pathogenesis of chronic inflammatory polyarthritis. Human cartilage glycoprotein-39 (HCgp-39), proteoglycan aggrecan (PG), and link protein (LP) can all, in certain situations, induce erosive polyarthritis in immunized animals.[12, 13, 16, 36] HCgp-39-derived peptides have been found to stimulate peripheral blood T cells from RA patients in vitro; they also bind with high affinity to the HLA-DR1 and -DR4 molecules associated with RA.[36] Likewise, patients with RA have been found with humoral or cellular[16] immunity to human PG. A preliminary indication of cellular immunity to human LP has appeared.[8]

These noncollagenous molecules also deserve consideration in the design of oral tolerance strategies for individual patients with chronic inflammatory arthritis. Nonetheless, the central candidate remains type II collagen. Of considerable interest is the recent elucidation by highly sophisticated radiograph crystal structure analysis that the HLA-DR4 (DRA*0101, DRB1*0401) peptide, which is unequivocally associated with RA susceptibility,[38] can intimately complex itself to a fragment of human type II collagen.[7] This observation furnishes independent support for the involvement of T-cell reactivity to type II collagen in the pathogenesis of RA and the therapeutic success achieved with type II collagen feeding in humans.[2, 33]

ANIMAL STUDIES

Beginning with collagen arthritis in rats[32] or mice[25], oral administration of type II collagen has been found to suppress virtually all experimentally inducible animal models that exist for RA.[35, 39] These systems include adjuvant arthritis, antigen-induced arthritis, pristane arthritis, streptococcal cell-wall arthritis, and silicone arthritis. In all protocols, the technique has been both potent and free of noticeable side effects. No discordance in clinical outcomes has surfaced between different laboratories involved in this work.

In all trials that have profiled dose-effect relationships of repetitive collagen feeding, a striking inverse association has been apparent.[39, 40] For example, in the adjuvant model, using rats weighing between 125 to 150 grams, daily doses of 3 or 30 μg of native chicken type II collagen abrogated the disease to a statistically significant degree, whereas higher doses or a dose of 0.3 μg/day were ineffective.[40] This narrow, low-dose window has also been noted and contrasted with more soluble antigen ranges in another rat system, the antigen-induced model.[39] Here, bovine serum albumin (BSA) is used as an intradermal immunogen, and the monoarticular disease is incited 14 days later by intra-articular instillation of BSA into the knee joint cavity of the now sensitized animal. Over the next few days, easily quantifiable knee joint swelling ensues. Daily oral doses of 3 or 30 μg of native chicken type II collagen again is suppressive in this system, whereas bioactivity is lost at other dose levels.[39] Feeding the inducing antigen, BSA, in this model is also effective at aborting joint swelling, but here the active dose range is much higher, approximating 1000 μg/day. The basis for this disparity is unknown but could relate to the highly insoluble nature of native type II collagen and its resistance to enzymatic degradation.[2, 4, 19, 34] Regardless of this conundrum, potential extrapolation in human trials was noticed. Here low,[1, 2, 33] but not high[29] collagen doses have been most effective.

MECHANISM OF ACTION

In arthritis models, there has not been a convincing explanation for why type II collagen feeding suppresses synovial inflammation. How-

ever, in other animal systems a number of studies have pinpointed two ways by which oral tolerance is likely to be achieved.[5, 11, 14, 15, 17, 20, 35, 37] Both pathways could operate independently or in a bimodal fashion. They are called *anergization* and *bystander suppression*. Anergization is an antigen-specific event whereby antigen committed T cells are converted to a state of physiologic dormancy, rather than activation, via antigen encounter. Exactly how this occurs is unclear but available evidence indicates that feeding high doses of antigen, particularly on an infrequent schedule, is necessary to trigger anergy. In marked contrast, feeding low doses of antigen, usually on a frequent repetitive basis, favors a response termed bystander suppression. Recognition that a dichotomy in CD4+ T cells exists, helper-T cells have been divided into Th-1 and Th-2 subsets.[17] Th-1 cells help to trigger and amplify immune and proinflammatory responses, whereas the Th-2 population appears to dampen both states. Th-1 and Th-2 orchestrations are mediated by specific cytokine release. Stimulatory molecules produced by Th-1 cells include interleukin-2 (IL-2) and interferon-γ (IFN-γ), whereas suppression is subserved by Th-2 release of transforming growth factor-β (TGF-β), IL-4, and IL-10. It appears that realignment of Th-1 and Th-2 effects is possible by oral antigen delivery and that low-dose feeding preferentially selects Th-2 cells for activation. The net down-regulatory effect is bystander suppression, since the process depends more on antigen-nonspecific release of a variety of immunosuppressive and anti-inflammatory cytokines.

A particularly straightforward protocol has been used to demonstrate anergy versus bystander suppression.[35] T cells from guinea pigs that were fed either a few high doses of ovalbumin or low doses on a frequent pattern around the time of immunization failed to proliferate to ovalbumin in vitro, showing that oral tolerance had been achieved since the expected outcome was a brisk response to antigen challenge. Addition of a monoclonal antibody specifically designed to eliminate TGF-β in the cultures restores the proliferative response of the low-dose derived T cells but leaves the nonresponsiveness of the high-dose group unchanged. This strongly implies that the low-dose oral tolerance is a function of bystander suppression (i.e., cytokine-mediated suppression), whereas anergy or some other process accounts for the inertness found in the high-dose group. That the high-dose oral tolerance is anergy is shown by the addition of IL-2 in vitro. This mediator reawakens the high-dose derived cells and leads to proliferation to ovalbumin. In contrast, the low-dose cells remain unresponsive to ovalbumin after the addition of IL-2.

PHASE I CLINICAL TRIAL OF TYPE II COLLAGEN FEEDING IN RA

Subsequent to finding an inverse dose relationship of collagen feeding in adjuvant arthritis, an open, 10-patient trial was designed to

initially assess the effect of feeding quite low doses of type II collagen in RA.[33] A dose escalation format was chosen to maximize the chance of a positive outcome. Specifically, a dose of 100 μg/day was to be given for a month, followed by 500 μg daily. Because collagen is an extremely insoluble protein, it was necessary to dissolve it in a dilute acetic acid. Solubilization was done at 4°C in order to preserve the native triple helical state of collagen.[34] To mask the vinegar taste of the solution and to maintain collagen solubility, at least to an extent, patients added the cold collagen solution to a glass of cold orange juice, which is also an acidic vehicle, immediately before ingesting on an empty stomach on arising in the morning. Smoking or beverage or food consumption was disallowed for at least the next 20 minutes.

The test collagen was in the form of highly purified fractions derived from chicken sternal cartilage. It had undergone ion exchange chromatography,[34] and was considered to be the optimal test antigen, based on a familiarity acquired during prior studies in the arthritis models and RA. Overlooked at the time, but of potential importance at present, is that the dose actually delivered to the patients was around two-thirds to three-quarters of the 100 and 500 μg parameters. For example, the lyophilized 100 μg collagen material that underwent solubilization had a water content of approximately 25% by weight as determined at a later time. Also additional small amounts of collagen were probably lost during the sterilization phase, which consisted of Millipore membrane passage prior to ingestion by the patient. Some patients appeared to improve during the open-label trial and others clearly worsened after discontinuing their disease-modifying, antirheumatic drugs (DMARDs), such as methotrexate. No side effects were observed setting the stage for a more rigorously controlled, 60-patient study.

In the subsequent Phase I double-blind trial,[33] the same 100 to 500 μg dose of collagen was compared to placebo, according to balanced assignment. Because of the appeal of a seemingly benign treatment, recruitment within a single center was accomplished. Candidates were carefully screened based on a goal of acquiring patients with active but manageable RA. In other words, patients were sought who were not DMARD refractory or had extremely severe RA because such flagrant patients might not respond to a relatively mild intervention. This aspect accounts for the relatively low joint counts at baseline,[33] compared to many other trials. Owed to the fact that it was still a pilot trial for feasibility, patients were merely taken off their DMARD and entered into the study without undergoing a washout period. From the outset the trial was intended solely to test the hypothesis that oral tolerization could be demonstrated in an established human autoimmune disease; measuring the exact efficacy of the procedure was a highly secondary objective.

Since a single investigator recruited and evaluated all patients, there was a considerable degree of homogeneity in the study, and on completion only a single patient was considered to be unevaluable. Trends favoring actual treatment were observed at the end of the

3-month trial, with improvement in the number of swollen joints and tender joints attaining statistical significance.[33] From a clinical standpoint, improvement in most patients was modest and no changes in the erythrocyte sedimentation rate (ESR) occurred. Since four patients in both the collagen and placebo groups improved to a remarkable extent, no evidence was obtained to show that a subset of patients with RA were susceptible to oral tolerization to collagen. Around 25% of the 60 patients had serum antibodies to type II collagen at baseline. Levels did not change with therapy and did not correlate with response to treatment. Rheumatoid factor and DR4 positivity also did not predict clinical outcome in this trial. No side effects were noted. It should not be overlooked, in retrospect, that appreciable treatment effects were found at 1 month of therapy and that minute hints of deterioration occurred in the next 2 months. It is uncertain whether less than 100 μg of collagen accounted for all the improvement in the 3-month trial.

This study is important in that it used scientifically accepted methodology to provide evidence of the validity of oral tolerization as an approach to the control of human arthritis. Being a novel proof-of-principle paper, it was submitted to a prestigious basic science journal for publication.[33] Unfortunately, it was overinterpreted by the lay public and the medical journalistic press in terms of its therapeutic implications.

THE POSITIVE MULTICENTER PHASE II TRIAL OF COLLAGEN FEEDING IN RHEUMATOID ARTHRITIS

Following data analysis of the 60-patient, single center study, a larger Phase II trial was designed to attempt to reproduce and extend these findings and, particularly, to address the deficiencies of the original trial.[2] Four major dimensions were incorporated into the new effort: (1) a dose-ranging aspect; (2) addressing the kinetics and durability of response by a 6-month treatment format; (3) including a DMARD washout to avoid potential ambiguity or confounding effects; and (4) larger scale assessment of the potential predictability of seropositivity for collagen antibodies and response to collagen treatment.

To accomplish these ambitious goals, it was necessary to enlist six geographically distinct sites. All had vast experience in RA trials and patients and personnel that were considered optimal to conduct the effort. Two hundred ninety seven patients were screened and 274 were randomized to receive in equal blocks, 20 μg, 100 μg, 500 μg, or 2500 μg of native chicken type II collagen or an identically appearing placebo solution. Patients were required to washoff DMARDs for 8 or 12 weeks prior to entry, depending on the type of medication. For the dominant DMARD, methotrexate, the interval was 8 weeks. To insure that treatment groups were balanced with regard to rheumatoid factor positivity, they were stratified by rheumatoid factor status.

Each group consisted of approximately 55 patients. Demographic and disease characteristics were similar in all five groups. Unfortunately, at final analysis, the duration of disease was considerable; the means

were between 10 to 13 years. Current and prior use of DMARDs and low-dose prednisone was quite heterogeneous. Two hundred twenty eight patients (83%) completed the 6-month treatment period. The frequency of dropouts was similar across all groups. Monthly assessments occurred. Overall compliance appeared to be satisfactory; protocol violations did not appear to confound treatment outcomes and no toxicity attributed to collagen was noted.

It was decided to use the Paulus criteria as the primary efficacy composite, since the American College of Rheumatology (ACR) criteria had been proposed but not validated in an independent trial at the time this multicenter study was designed. Using this instrument in an intent-to-treat format, there was a numerically uniform inverse dose-response relationship to collagen feeding. The 39% ever-response rate to 20 μg of collagen was significantly ($p < 0.05$) superior to the placebo rate of 19% and each higher dose produced fewer responders (range 33% to 24%). Additional trends favoring the 20 μg dose were also observed but because of the low statistical power afforded by the small individual group size, none achieved statistical significance.

At baseline, serum IgA and IgG antibodies to type II collagen were detected in 37% and 18% of the patients, respectively. Comparing the group that received any dose of collagen with the placebo group showed that the presence of collagen antibodies was significantly associated with the likelihood of responding to collagen treatment, as judged by the Paulus criteria ($p < 0.02$ for IgA seropositivity and $p < 0.05$ for IgG seropositivity). Of further importance, antibody profiles did not change with treatment, indicating, on a large scale measure, that sensitization to collagen or suppression of collagen immunity did not occur.

Potential criticisms and weaknesses of this work are discussed in Barnett et al.[2] Overall, the positive outcome is noteworthy for several reasons. Most importantly, it is the first substantive indication that oral tolerance can be used to modify established autoimmune disease. In addition, the approach appears to be devoid of side effects. Although the linkage of seropositivity for collagen antibodies with responsiveness to collagen feeding is only tentative, it is an observation that warrants further assessment in future trials.

The low doses of collagen found to be effective in the Phase I[33] and II[2] trials parallel work in animal systems,[39, 40] and suggest that inhibitory Th-2 cytokine release is operative in the human sphere. Although the degree of clinical improvement observed in both trials was modest, it was accomplished in the absence of background DMARD administration. While optimal for trial design, collagen monotherapy fails to address the potential of collagen treatment in a combination approach for RA.

AN ADDITIONAL CLINICAL TRIAL

Another well-conducted, double-blind, randomized, placebo-controlled trial of type II collagen therapy has been published.[29] Sieper et

al. in Berlin were able to enroll 90 patients with early RA (disease duration <3 years) into a 3-month study.[29] Thirty patients each received native bovine type II collagen at a daily dose of 1 mg or 10 mg and the final 30 were randomized to placebo. Fifty percent to 60% of the patients were rheumatoid factor positive and the majority had not received prior DMARD therapy. DMARDs were not allowed during the trial. Even at these high doses, collagen was well tolerated. Evaluation at 3 months showed that three patients in the 10 mg group had a response characterized as marked; 1 in the 1 mg dose limb had a response of similar magnitude, and no patients responded in the placebo group. Fourteen patients dropped out of the study, 12 of which were adjudged to reflect lack of efficacy. Collagen antibody status was not addressed in this article.[29]

At initial perusal, this would have to be considered a negative result. But the authors seemed to be impressed by the degree of benefit exhibited in the extremely small subset of patients. The outcome parallels the lack of overall improvement in the 54 patient cohort treated with 2500 μg in the 6-month multicenter study.[2] It is also compatible with the hypothesis that high dose collagen feeding, as in animal systems,[35, 37] creates a state of T-cell anergy. The improvement in 4 of 60 patients treated with collagen could mean that autoimmunity to type II collagen is a fundamental element in a small fraction of patients considered, at present, to have RA.

CURRENT STATUS OF COLLAGEN INGESTION

A number of aspects should be addressed regarding the present status and future directions for oral tolerance therapy in RA. Of greatest current concern is the indiscriminant use of collagen in this disorder. One form of malfeasance is culinary attempts by patients to extract collagen from, for example, pigs' knuckles by boiling, semipurification by passage through cloth or coffee filters, followed by ingestion of the denatured protein in the broth. At the other extreme are companies in the United States and United Kingdom that are selling preparations purporting to contain type II collagen as a remedy for arthritis. Tabloid advertisements of efficacy have appeared on a wide-scale basis. Although technically this is a violation of the law, the US Food and Drug Administration (FDA) has, to date, been effete in acting to curtail these practices. As well as condemning the practice as medical charlatanism, the author has two additional concerns. Independent of the unregulated nature of the putative collagen's content and degree to which its native triple helical structure has been maintained, overdosing of collagen, based on current evidence,[2, 29] would in all likelihood be ineffectual. Theoretically, ingesting high doses of collagen could lead to a long-lasting "de-tuning" of the body's gut-associated lymphoid tissue (GALT) to subsequent collagen ingestion. Attempts in the future to generate oral tolerance to collagen might be thwarted. A final caveat of concern is that

unrecognized bacterial, viral, or even prion contamination could be present in animal extracts. Bovine spongiform or "mad cow disease" illustrates this potentiality. Available technology makes it more cost effective to use constituents from bovine material. Chicken cartilage could also contain *Salmonella* or avian viruses. To the consumer and physician advocate, caveat emptor.

FURTHER DEVELOPMENTAL DIRECTIONS

In the author's opinion, sufficiently compelling information has been acquired for native chicken type II collagen to enter final Phase III testing for a new drug application (NDA) to the FDA. This judgment is based, in large part, on an unprecedentedly safe risk-benefit ratio for a treatment for RA. Treatment tolerability is notoriously poor in RA. Even a compound with modest effectiveness would be useful.

Testing should not exclusively involve heterologous collagen. Bovine preparations, because of mad cow disease, cannot tacitly be assigned as harmless. It is methodologically possible to generate recombinant human analogues of type II, IX, and XI collagens. The proper treatment of humans may be humans, and perhaps homologous preparations would be more effective. In addition, there is a decided variance in an individual RA patient's antibody repertoire to collagen epitopes. Conceivably certain patients might respond better to type IX or XI collagen than to type II. What about combined collagen feeding?

Along these lines, other cartilage constituents should be considered. As outlined previously, noncollagenous moieties, such as HCgp-39, PG, and LP are arthritogenic in experimental systems and deserve scrutiny in the oral treatment of a variety of inflammatory arthritis conditions, including psoriatic arthritis and ankylosing spondylitis as well as RA.

Finally, an extremely small uncontrolled trial[1] suggests that oral type II collagen can ameliorate juvenile rheumatoid arthritis (JRA). In view of its tolerability, what more compelling indication for subsequent clinical testing could exist? For better or worse, the commercial marketplace drives treatment development. Support by the National Institutes of Health (NIH) traditionally stops after the preclinical therapeutic stage. Orphan diseases, such as JRA, continue to be underaddressed by the scientific and pharmaceutical communities. Will this practice ever change? Probably not unless vigorous lay and political pressure is applied.

CONCLUSION

Currently, the basic science immunology community is quite accepting of the phenomenon of oral tolerance induction in animals. The only unanswered questions are the pathway(s) by which it occurs, ways in which it can be amplified,[15, 41] and whether it works for estab-

lished/preexistent immunity. Accordingly, further studies using cytokine supplementation, cholera toxin, and lipopolysaccharide (LPS) are underway.

In contradistinction, the clinical community is, not surprisingly, somewhat agnostic regarding oral tolerance. Progress in multiple sclerosis has not been definitive and outcomes in RA have been modest at best. Recent reports in animal models have suggested that oral ingestion of autoantigen can have deleterious effects upon the host. Although those experiments have had a highly artificial or manipulated framework, they are consistent with the possibility that oral antigen therapy in human disease may be (1) beneficial; (2) of no consequence; or (3) detrimental. An extremely open mind will hopefully be applied to future research efforts.

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Immunology

Systemic versus cartilage-specific expression of a type II collagen-specific T-cell epitope determines the level of tolerance and susceptibility to arthritis

(collagen-induced arthritis/T-cell tolerance/transgenic mice/autoimmunity/rheumatoid arthritis)

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ABSTRACT **Immunization of mice with rat type II collagen (CII), a cartilage-specific protein, leads to development of collagen-induced arthritis (CIA), a model for rheumatoid arthritis. To define the interaction between the immune system and cartilage, we produced two sets of transgenic mice. In the first we point mutated the mouse CII gene to express an earlier defined T-cell epitope, CII-(256–270), present in rat CII. In the second we mutated the mouse type I collagen gene to express the same T-cell epitope. The mice with mutated type I collagen showed no T-cell reactivity to rat CII and were resistant to CIA. Thus, the CII-(256–270) epitope is immunodominant and critical for development of CIA. In contrast, the mice with mutated CII had an intact B-cell response and had T cells which could produce γ interferon, but not proliferate, in response to CII. They developed CIA, albeit with a reduced incidence. Thus, we conclude that T cells recognize CII derived from endogenous cartilage and are partially tolerized but may still be capable of mediating CIA.**

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease affecting peripheral joints. The main genetic association is to the major histocompatibility complex (MHC) class II region (HLA-DR) (1, 2), suggesting involvement of T-cell-mediated autoimmune recognition of joint-specific antigens. Known proteins within joints that can be considered joint-specific are those found in articular cartilage. These include collagens of type II (CII), type IX (CIX), and type XI (CXI), and noncollagenous proteins such as aggrecan and COMP (3–5). Some of these cartilage-specific proteins (CII, CXI, and aggrecan) have been shown to induce autoimmune arthritis in animals (6–8). The most widely used model is collagen-induced arthritis (CIA), induced with CII, the major protein component of cartilage. Immunization with CII leads to development of CIA in rats, mice, and apes (6, 9, 10). In mice, the disease has a higher incidence and severity after immunization with heterologous CII compared with after immunization with autologous CII (11). The immune recognition of CII, and the subsequent arthritis development, is surprisingly precise. The disease is associated with the expression of the MHC class II A^q molecule (12). One limited region of CII, between positions 256 and 270, is recognized after immunization with rat CII (13). This peptide is present in heterologous but not in mouse CII and can be recognized both in its posttranslational modified form (disaccharides bound to hydroxylysines) and in its nonmodified form (14). The difference is due to the presence of an aspartic acid at position 266 in mouse CII, whereas a glutamic acid is present at that position in rat CII. The lack of crossreactivity could possibly be explained by the finding that the rat CII-(256–270) binds better than the mouse peptide to the MHC class II A^q molecule (13). Still, autoreactive peptide-specific T cells can be activated, but only after immunization with mouse CII-(256–270).

In recent years there has been considerable progress in the understanding of the basic mechanisms for development of immune tolerance to self-antigens. Elimination of self-reactive T cells in thymus (central tolerance) has been clearly demonstrated (15) and autoreactive T cells which are not eliminated in the thymus may be eliminated or develop tolerance if exposed to autoantigens in the periphery (16, 17). However, in other cases T cells respond to exposed autoantigens (18). For the study of tolerance in autoimmune disease the nature of the antigen and the context by which it is presented is of critical importance. Thus, it is essential that disease-related autoantigens are studied. The expression of the CII gene is tightly controlled in a tissue-specific fashion through regulatory elements located in the promoter and first intron (19, 20). We have hypothesized that T cells specific for CII in cartilage are not eliminated but are anergized and may play an important role in the development of CIA (11). To address this question we made mice transgenic with type II and type I collagen genes which were mutated to express the immunodominant CII-(256–270) epitope. The behavior of CII-reactive T cells and arthritis susceptibility in these mice were analyzed.

MATERIALS AND METHODS

Animals. C3H.Q mice (originally from D. C. Shreffler, Washington University, St. Louis), were bred, kept, and used in environmentally controlled but conventional animal facilities.

Transgene Constructs. For cartilage-specific expression, a 40-kb genomic clone of the mouse CII gene (*Col2a1*) was used, containing the entire gene, including flanking sequences (21). This clone has earlier been shown to direct cartilage-specific expression in transgenic mice (22). By site-directed mutagenesis, we introduced one nucleotide mutation leading to an amino acid shift from Asp to Glu (Fig. 1*A*). Syngeneically fertilized C3H.Q eggs obtained from superovulated donors were subjected to pronuclear microinjections. Two founder

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Abbreviations: CIA, collagen-induced arthritis; CI, type I collagen; CII, type II collagen; MHC, major histocompatibility complex; RA, rheumatoid arthritis; MMC, mutated mouse collagen; TSC, T-cell epitope in systemic collagen; RT, reverse transcriptase; LNC, lymph node cells; IFN-γ, γ interferon; ELISPOT, enzyme-linked immunospot; PPD, purified protein derivative.

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lines, MMC-1 and MMC-2 were obtained (MMC, mutated mouse collagen).

To achieve systemic expression of CII-(256–270) in a chimeric type I collagen (CI), a derivative of the mouse genomic clone 10D of the *Col1a1* gene (kindly provided by Hong Wu, Whitehead Institute, Boston) was used for site-directed mutagenesis. Complementary oligonucleotides covering the CII-(256–270) coding sequence were synthesized for replacement of a casette of wild-type sequences in exon 43 of the *Col1a1* gene (Fig. 1*B*). The 26.6-kb construct has been successfully expressed in 3T3 fibroblasts and the product has been shown to stimulate CII-specific T-cell hybridomas (14). This construct was similarly used for microinjection of fertilized C3H.Q eggs. Two founders were generated: TSC-1 and TSC-2 (TSC, T-cell epitope in systemic collagen).

Screening and Transgenic Expression. Genomic DNA was prepared from the tip of the tail or a toe (23). MMC DNA was screened by using PCR with CII-specific primers and a transgene-specific restriction digest (*Bcl* I silenced by the point mutation) followed by agarose gel electrophoresis. TSC DNA was screened by using PCR with CI-specific primers and a transgene-specific restriction digest (*Sty* I introduced in transgenic TSC mice). The negative, nontransgenic, littermates (C3H.Q) were used as control mice in all experiments. The copy numbers of integrated MMC constructs were determined by Southern blotting; MMC-1 has approximately six inserted copies, while MMC-2 has nine (data not shown).

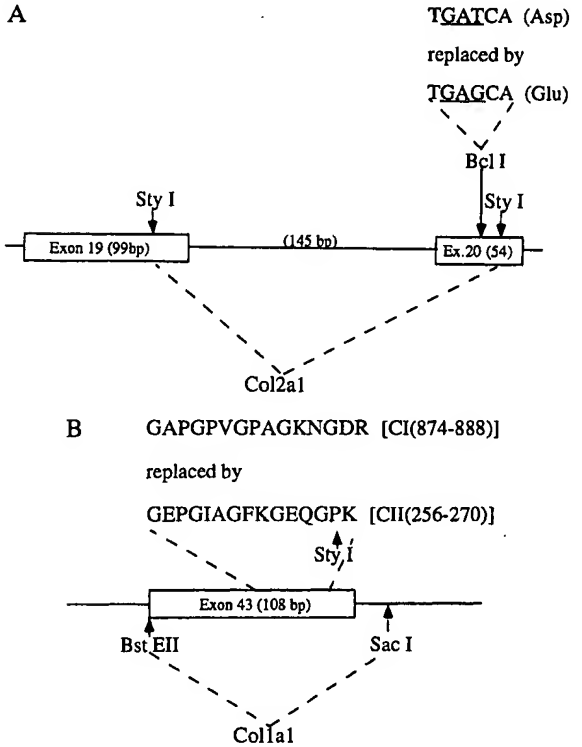

FIG. 1. (*A*) Gene construct for mutant CII: MMC transgenic mice. By PCR, the reverse primer 5′-TCTCCCTTGGGGCCTTGCTCACC-3′ introduced the desired mutation, and at the same time silenced a *Bcl* I restriction site, which was later used for transgene identification. The mutated sequence replaced the wild-type sequence between the *Sty* I restriction sites. (*B*) Gene construct for systemic CII-(256–270) expression: TSC transgenic mice. Synthetic oligonucleotides, 113 and 122 nt, were annealed to recreate a mutant exon 43, now encoding the CII-(256–270) epitope. Restriction enzymes *Bst*EII and *Sac* I were used to replace the wild-type sequence.

Reverse Transcriptase (RT)-PCR. Tissue was frozen in liquid nitrogen and kept at −85°C until total RNA was prepared (24). Transgene-specific primers were used in the cDNA reactions, which were followed by PCR and agarose gel electrophoresis.

Collagen Preparations. Rat CII was extracted from the Swarm chondrosarcoma (25) after pepsin digestion or from lathyritic chondrosarcoma (26) and further purified as described (27). The collagen was dissolved and stored in 0.1 M acetic acid until used. Collagen was also extracted by pepsin digestion from skin and the xiphoid process of transgenic mice and their nontransgenic littermates by the same method but without further purification. CII-(256–270) was synthesized as described (13).

Induction of Arthritis. Male mice, 7–12 weeks of age, were immunized intradermally in the base of the tail with 100 μg of CII emulsified in complete Freund's adjuvant (Difco). They were also bled and given a boost injection with 25–50 μg of CII emulsified in incomplete Freund's adjuvant (DIFCO) at day 35. The amount of CII-reactive antibodies in the sera was measured by a quantitative ELISA (28) and clinical scoring was performed as described (11).

Immune Response Assays of Primary Cultures. Age- and sex-matched mice were immunized in the footpads with 50 μg of lathyritic rat CII emulsified in complete adjuvant. Draining lymph nodes were collected 10 days later and single cell suspensions were prepared in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% fresh mouse serum, Hepes, penicillin, streptomycin, and 2-mercaptoethanol.

To measure the antigen-specific proliferative response, the lymph node cells (LNC) were cultured in flat-bottomed 96-well plates (Nunc, Roskide, Denmark) and stimulated with antigen for 96 hr before pulsing with [^{3}H]thymidine and were harvested 15–18 hr later in a Filtermate cell harvester (Packard). The incorporation of [^{3}H]thymidine was determined in a Matrix 96 Direct Beta Counter (Packard); all experiments were performed in triplicate cultures.

The presence of immunoglobulin as well as γ interferon (IFN-γ)-producing cells was determined by enzyme-linked immunospot (ELISPOT) assays essentially as described (29). In the ELISPOT assay for enumeration of anti-CII immunoglobulin-secreting B cells, 96-well plates were coated with mouse CII or CI at 20 μg/ml and 5×10^5 LNC were added per well and incubated for 1–2 hr. After removal of the cells a peroxidase-conjugated anti-mouse immunoglobulin antibody was added. Plates were developed by using 3-amino-9-ethylcarbazole (Sigma). In the IFN-γ ELISPOT assay, 96-well nitrocellulose plates (Millipore) were coated with a rat anti-IFN-γ antibody. LNC from a 48-hr primary culture of 10^6 cells per well were added to the plate and incubated for 24 hr. After the cells had been removed, anti-IFN-γ rabbit immunoglobulin, biotinylated anti-rabbit immunoglobulin, and avidin-biotin-peroxidase (ExtraAvidin, Sigma) were added before developing with 3-amino-9-ethylcarbazole. Spots were counted in a microscope in both ELISPOT assays. Mean values of duplicate or triplicate cultures were used.

Assay for Detection of Transgenic Collagen. Collagen preparations from skin and the xiphoid process of the different mice were used to stimulate the CII-(256–270)-specific T-cell hybridoma HCQ.10 (14). This hybridoma is extremely sensitive to glycosylated CII and reacts at the level of 1 ng/ml; no crossreactions to nonglycosylated CII have been seen. To detect the presence of transgenic collagen containing the CII-(256–270) region in the various collagen preparations, 5×10^4 HCQ.10 cells were incubated with collagen and 5×10^5 C3H.Q splenocytes as antigen-presenting cells in 96-well plates for 24 hr, whereafter the plates were frozen. The interleukin 2 content was determined by using the cytotoxic T-lymphocyte line (CTLL). In this assay 10^4 CTLL cells were incubated with

supernatant for 24 hr before pulsing and harvesting as described for the immune response assay.

Statistics. Dichotomous variables (i.e., incidence of arthritis) were analyzed by χ^2 test, and nonparametric (i.e., arthritic scores) or values with a skewed distribution (e.g., stimulation indices and antibody levels) were analyzed by the Mann–Whitney *U* test.

RESULTS

Generation of Transgenic Mice Expressing Mutated Collagens. To express the immunodominant CII-(256–270) epitope in CII, we used a 40-kb mouse CII genomic construct. By PCR one point mutation (T to G) was created, leading to the desired amino acid change from Asp to Glu at position 266 (Fig. 1*A*).

To express the CII-(256–270) epitope in systemically occurring CI, a mouse *Col1a1* genomic clone containing all the regulatory elements for tissue-specific expression of CI, was mutated (Fig. 1*B*). The replacement mutations of 9 amino acid residues remained restricted to the X and Y positions of the collagen-specific Gly-X-Y sequence, thus leaving the glycine residues in every third position unchanged as a critical structural requirement for triple helicity of the native chimeric collagen.

The above constructs were introduced as transgenes into the CIA susceptible mouse strain C3H.Q. Two transgenic lines carrying the mutated CI gene (TSC-1 and TSC-2) and two lines carrying the mutated CII gene (MMC-1 and MMC-2) were founded and maintained as heterozygotes. Only one of each (TSC-1 and MMC-1) was used, since the founders for each construct showed similar types of expression and immune responses. None of the mice have developed arthritis spontaneously and no other macroscopic disturbances in breeding or development of the mice have been noted. This is in contrast to CII-transgenic mice mutated at positions coding for glycines, or mice with truncated CII, which develop chondrodysplasias of varying severity (22, 30, 31). Similarly, CI-transgenic mice with mutated glycines develop diseases such as osteogenesis imperfecta (32).

Total RNA was prepared from newborn transgenic MMC-1 and TSC-1 mice as well as from nontransgenic littermates for the analysis of transgene-derived mRNA by RT-PCR from the following tissues; eye, thymus, liver, kidney, spleen, skin, and the sternal xiphoid process (cartilage). Transgenic mRNA could be detected only in the eye and cartilage of MMC-1 mice (Fig. 2), in accordance with the strict tissue-specific expression of the CII gene (19, 20), although illegitimate CII mRNA has been observed in many organs from normal mice by using RT-PCR under conditions apparently better than those presently used (30, 33). TSC-1 transgenic mRNA was detectable in all of the above-mentioned samples (data not shown). To analyze whether transgenic protein was actually expressed, collagen was prepared from skin and the sternal xiphoid process from the two different lines of mice. The collagen was used for stimulation of a CII-(256–270)-specific T-cell hybridoma (14) recognizing the glycosylated form of the peptide (Fig. 3). Collagen prepared from the skin and xiphoid process of TSC-1 mice and collagen prepared from the xiphoid process of MMC-1 mice contained transgenic protein. These data show that the expression of transgenic collagens is posttranslationally modified and restricted in accordance with the tissue specificity of the corresponding wild-type gene expression in the adult mouse.

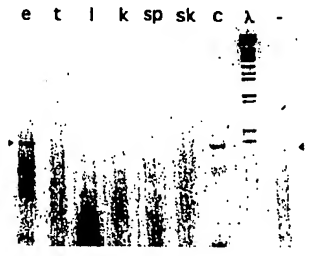

FIG. 2. Expression pattern of transgenic CII in MMC-1 mice. Expression of mutant *Col2a1* mRNA in tissues of newborn MMC transgenic mice analyzed by RT-PCR and gel electrophoresis. From left to right: eye, thymus, liver, kidney, spleen, skin, and cartilage (paw). λ, Size marker *Eco* 471-digested phage λ DNA. As a negative control, total RNA from a paw of a C3H.Q mouse was used. The product is 501 bp and is indicated by arrowheads.

TSC-1 Mice Developed no Immune Response to Rat CII. Draining lymph node cells from TSC-1 mice immunized with rat CII failed to mount a significant immune response to CII-(256–270) as measured with IFN-γ ELISPOT assays (Fig. 4) or proliferation (Table 1). This suggests that the responding T-cell population has been eliminated or at least behaves as if functionally dead. In fact, also after stimulation with the entire rat CII, no or very poor T-cell responses were recorded. In addition, T-cell help to B cells was lacking, since B cells were not, or were very poorly, activated into anti-CII immunoglobulin secretion as measured with an ELISPOT assay (Fig. 5). The relative lack of immune response to rat CII suggests that

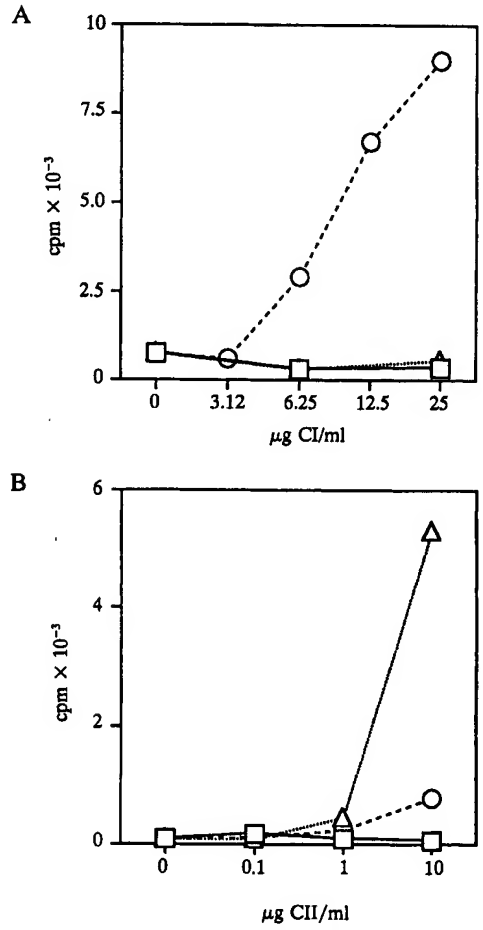

FIG. 3. Detection of transgenic collagen. CTLL assay with T-cell hybridoma HCQ.10 specific for the glycosylated CII-(256–270) epitope. (*A*) Stimulation with CI (skin collagen) preparations from C3H.Q (□), MMC-1 (△), and TSC-1 (○). (*B*) Stimulation with CII (xiphoid cartilage collagen) preparations from C3H.Q (□), MMC-1 (△), and TSC-1 (○).

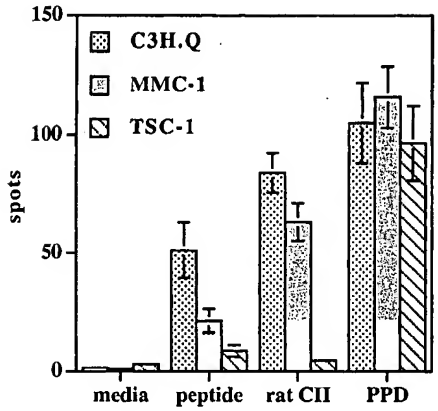

FIG. 4. Number of IFN-γ-producing T cells after CII immunization. IFN-γ ELISPOT assay on cells from draining lymph nodes of mice immunized with rat CII. The LNC were stimulated with 100 μg of rat CII per ml, 50 μg of CII-(256–270) per ml, or 10 μg of purified protein derivative (PPD) per ml. The results are expressed as number of spots per 10^6 LNC. The graph summarizes several experiments, each with balanced groups. In total, 23 C3H.Q, 10 MMC-1, and 9 TSC-1 mice were used. Error bars indicate SD of the biological variation.

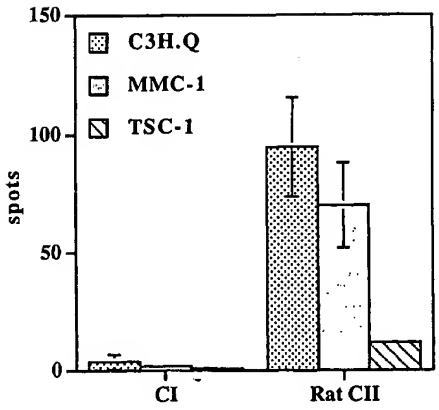

FIG. 5. Number of anti-CII reactive B cells. ELISPOT assay of immunoglobulin on cells from draining lymph nodes of mice immunized with rat CII. The results are expressed as number of spots per 0.5×10^6 LNC. The graph summarizes several experiments, each set up with balanced groups. In total, 8 C3H.Q, 6 MMC-1, and 2 TSC-1 mice were used. Error bars indicate SD of the biological variation.

epitopes other than CII-(256–270) only very marginally contribute to the anti-CII immune response. In fact, this immune response is at the same level as the response to mouse CII (27), which has a cryptic CII-(256–270) peptide (13) as well as a few other weakly stimulatory peptides in other regions in the mouse CII molecule (34).

MMC-1 Mice Mount a Partially Tolerized T-Cell Response to CII. Draining lymph node cells from MMC-1 mice immunized with rat CII mounted a reduced but still significant response to both CII-(256–270) and rat CII compared with C3H.Q when measured with the IFN-γ ELISPOT assay (Fig. 4). In addition, mice immunized with rat CII developed normal levels of anti-CII B-cell response as quantitated with the immunoglobulin ELISPOT assay (Fig. 5). However, no significant proliferative responses to CII-(256–270) or to the entire rat CII could be found (Table 1). These findings show that the CII-(256–270)-reactive T cells were not eliminated but tolerized.

MMC-1 Mice Develop CIA, but with a Reduced Incidence. Immunization with rat CII induced in all groups of nontransgenic littermates (C3H.Q) a high incidence of severe arthritis (Fig. 6). None of the TSC-1 mice developed arthritis. MMC-1 mice, on the other hand, developed CIA, and the mice had as severe disease as the control group, but the incidence was reduced. The antibody response showed no significant difference between C3H.Q and MMC-1 (Fig. 7). The TSC-1 mice, on the other hand, did not develop significant levels of anti-mouse CII antibodies. Even 2 weeks after boost (day 49) the antibody levels were negligible (14 ± 12 μg/ml) compared with those of C3H.Q (450 ± 185 μg/ml).

Table 1. Comparison of the stimulation index in the proliferative response after CII immunization

Mice	Stimulation index			
	CII-(256–270)	*P*	Rat CII	*P*
MMC-1*	1.87 ± 0.66	0.0025	1.71 ± 1.17	0.0248
C3H.Q	6.09 ± 4.06		6.65 ± 6.83	
TSC-1†	1.01 ± 0.22	0.0086	1.16 ± 0.55	0.1197
C3H.Q	2.51 ± 1.40		1.92 ± 1.27	

Proliferative response to CII-(256–270) and rat CII from MMC-1, TSC-1, and C3H.Q mice immunized with lathyritic rat CII (±SD). The stimulation index is calculated as proliferation at the highest antigen concentration [100 μg of rat CII per ml and 50 μg of CII-(256–270) per ml, respectively] divided by the background proliferation (no antigen added). Each experiment was set up in duplicate or triplicate cultures; the methodological variation was less than 15%, and the PPD responses were comparable within the groups. The SD thus illustrates the biological variation in our mice.

*Summary of six experiments comparing 11 MMC-1 mice with 15 nontransgenic littermates.

†Summary of four experiments comparing 10 TSC-1 mice with 13 nontransgenic littermates.

DISCUSSION

In the present experiments we show that autoreactive CII-specific T cells behave differently, depending on whether the recognized epitope, CII-(256–270), is located in cartilage or expressed in noncartilaginous matrices. In the latter case, the CII-(256–270) epitope was incorporated in the CI gene and transgenically expressed in the TSC-1 mouse. The lack of T cells responding to CII-(256–270) in TSC-1 mice indicates that the immune system recognizes collagen located in systemically

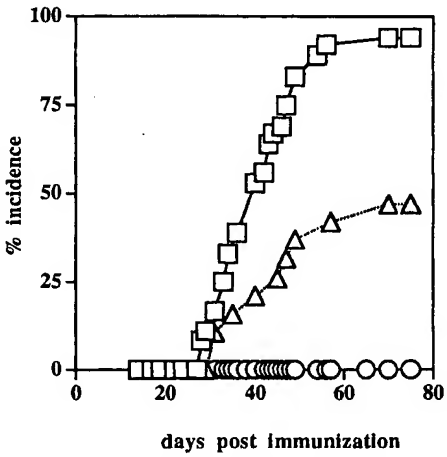

FIG. 6. Incidence (%) of arthritis. All mice were immunized at day 0 and boosted at day 35. The figure represents three experiments, each with balanced groups of male mice. In total, 25 C3H.Q mice (□), 20 MMC-1 mice (△), and 6 TSC-1 mice (○) were used. The incidence of arthritis differed significantly among all groups ($P = 0.0008$), while the severity of disease did not (6.1 ± 2.1 for C3H.Q and 5.0 ± 2.3 for MMC-1 mice).

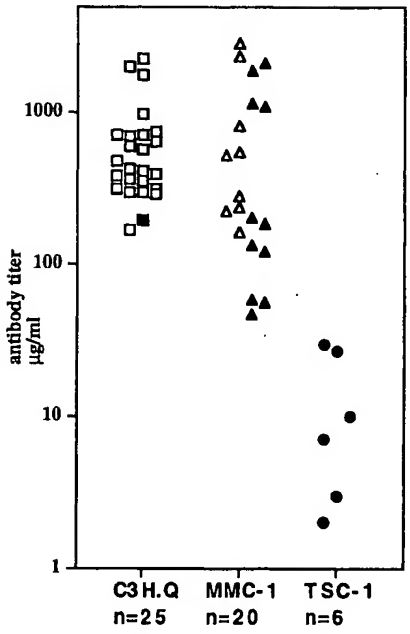

FIG. 7. Levels of mouse anti-mouse CII antibodies. Mice in the arthritis experiments were bled at day 35 after immunization, and the sera were used to measure levels of anti-mouse CII antibodies. Open symbols represent animals with arthritis and filled symbols, mice without disease. The mean levels of C3H.Q and MMC-1 anti CII antibodies did not differ significantly, while TSC-1 mice had significantly reduced antibody levels ($P = 0.0003$).

distributed connective tissue and therefore the responding T cells are physically or functionally eliminated. No T-cell response could be detected towards the entire rat CII either, indicating that there is no other immunodominant T-cell epitope in rat CII.

The insertion of the CII-(256–270) epitope into mouse CII required only one point mutation in the gene, and the localization of the gene product showed the predicted cartilage restriction in the transgenic MMC-1 mouse. The activation of IFN-γ secretion by the peptide-specific T cells, and the occurrence of functional help to B cells, clearly demonstrate that the CII-(256–270)-specific T cells have not been clonally eliminated and that the CII-specific T cells retain important effector functions. However, the antigen-specific proliferative response after stimulation *in vitro* was severely decreased. This is fully compatible with the definition of anergy as put forward by Schwartz and Jenkins: a poor proliferative capacity while effector functions are retained (35). The higher requirements to trigger activation and the failure to expand after activation could explain why the mice do not spontaneously develop arthritis. In addition, many other genetic and environmental factors may influence a possible outbreak of spontaneous arthritis. Thus, we have recently observed that an influence of stress (induced by intermale aggressiveness) in certain mouse strains, such as DBA/1, both "spontaneously" trigger arthritis (36) and lead to enhanced development of CIA (L. Jansson, personal communication). The C3H.Q strain is relatively resistant to development of stress induced arthritis (unpublished observations).

The reduction in susceptibility to arthritis in the MMC-1 mice clearly shows that the tolerant state of CII-(256–270)-reactive T cells is protective. It also shows that activation of only B-cells, with subsequent production of antibodies to native CII, is not sufficient for development of CIA. There is no doubt that antibodies play an important pathogenic role in CIA (37) but, for development of the complete disease, activation of autoreactive T cells appears to be critical.

The most pertinent but unsolved question is why 45% of the MMC-1 mice still developed severe arthritis. Is the arthritis mediated by partially tolerant T cells with retained effector functions or is it mediated by T cells which have escaped tolerance induction, perhaps by recent export from the thymus? The poor proliferative responses after secondary stimulation *in vitro* indicate that these T cells must be few, if any, which argues against the latter possibility. On the other hand, this possibility cannot be excluded, especially since collagens in general are problematic to handle for certain professional antigen-presenting cells and therefore usually give rise to relatively low T-cell proliferative responses *in vitro* (38). Another possibility is that the mutated CII is not properly posttranslationally modified, which is important because glycosylated CII is more arthritogenic than the nonglycosylated CII (14). However, in the present experiment we could directly show that glycosylated peptides are presented in both types of transgenic mice, since the collagen preparations stimulated a T-cell hybridoma reactive only to glycosylated CII-(256–270). An alternative explanation could be a shift to another cytokine secretion pattern—i.e., to a TH2-like immune response. The secretion of IFN-γ by the responding T cells strongly argues against this. Moreover, the possibilities for an active anticlonotypic regulation are limited, since CII-(256–270) is recognized by T cells using a very diverse set of T-cell receptors (A. Corthay, personal communication). Thus, an interesting remaining possibility is that it is in fact the partially tolerant T cells which mediate the arthritis. We imagine that there is a pool of CII-reactive T cells with variable degrees of tolerization depending on their antigen-interacting history. After immunization, the degree of proliferative activity ranges from full activation of T cells that have not earlier encountered the antigen to more or less tolerized T cells with limited proliferative activity, due to lack of interleukin 2 secretion.

These findings bear on the elucidation of autoimmune mechanisms in RA. The immunodominant CII-(256–270) peptide is present in human CII. In addition, the MHC class II molecule binding the CII-(256–270) peptide in the mouse, the A^q molecule (12), has striking similarities (11, 44) with the DR4 class II molecules in humans thought to be associated with RA (2). Moreover, B cells producing antibodies to CII are frequently found in the joints of RA patients, and their occurrence has been shown to be associated with DR4 (39–41). In contrast, in spite of considerable efforts by many investigators, only very few examples of CII-reactive human T cell clones have been reported (42, 43). A possible explanation is that in individuals with DR4 alleles the T cells are tolerized and thereby difficult to clone. Maybe they still maintain important effector functions (such as B-cell help and IFN-γ production) if they are stimulated by some environmental challenge which will expose the CII-(256–270) peptide or a mimicking foreign peptide in an immunogenic fashion.

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